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13. ABSTRACT (Maximum 200 Words)

Psoriasin (S100A7) is expressed at low levels in normal breast epithelial cells but is highly expressed in preinvasive ductal carcinoma in situ. Persistent psoriasin expression occurs in some invasive carcinomas and is associated with poor prognostic factors. We have found that psoriasin physically interacts with Jab1 (c-jun activation-domain binding protein 1) in the yeast two-hybrid assay and confirmed this by coimmunoprecipitation assay in breast cancer cells. Psoriasin-transfected breast cancer cells showed increased nuclear Jab1 and demonstrated several features consistent with an alteration in Jab1 activity including an increase in activator protein-1 (AP-1) activity, increased expression of AP-1 and HIF-1-dependent genes, and reduced expression of the cell-cycle inhibitor p27^{Kip1}. Psoriasin overexpression was also associated with alteration of cellular functions that are associated with increased malignancy, including increased growth, decreased adhesion, and increased invasiveness in vitro, as well as increased tumorigenicity in vivo in nude mice. We conclude that intracellular psoriasin influences breast cancer progression and that this may occur through stimulation of Jab1 activity.

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Introduction

We have previously identified the psoriasin gene (S100A7) as highly expressed in pre-invasive breast cancer and subsequently found that persistent psoriasin expression in invasive breast cancer is associated with markers of poor prognosis. The S100 proteins are calcium-binding proteins that may influence several cellular activities such as growth, chemotaxis, adhesion and cytoskeletal activity. The function of psoriasin and its influence in breast cancer is unknown. However *in vitro* and *in vivo* study of its role in psoriasis and also in breast tumors suggests that one possible role may be to influence the inflammatory response as a secreted chemotactic factor. We and others have also observed nuclear and cytoplasmic expression within squamous and breast epithelial cells, which raises the possibility of an additional function within epithelial cells. We therefore sought to pursue a possible intracellular function by utilizing the yeast 2-hybrid approach to screen a normal human breast expression library for intracellular breast epithelial proteins that might interact with psoriasin and that might offer insights into its functional role in breast tumor progression. We have identified two proteins that psoriasin can potentially form an interaction with, RanBPM and Jab1. The aim was to understand the importance of psoriasin expression in pre-invasive disease as well as any molecular mechanisms that it may operate through to exert its effects on the process of invasion. Through these studies we have found psoriasin to be associated with Estrogen Receptor negative breast cancer and is associated with a shorter time to recurrence of disease and poorer patient survival. We have also found psoriasin to influence the activity of the multifunctional protein Jab1 in breast cancer cells resulting in a more aggressive behaviour *in-vitro* and *in-vivo*.

Body

Original Statement of Work

AIM 1.

1-6	Construct RanBPM and S100A7 – epitope tagged expression constructs
6-12	Conduct transient transfection assays with RanBPM and S100A7 to co localize using confocal microscopy and study biochemical interaction by immunoprecipitation

AIM 2

1-6	Construct RanBPM expression constructs for inducible stable transfection (T Rex system vectors)
6-12	Stable transfection of MCF7 cell line
12-36	Analysis of effect of overexpression to RanBPM on growth, polarity, motility and invasion.

AIM 3

1-6	Construct RanBPM in-situ hybridisation probes for tissue analysis
12-36	Examine cell type, localization and level of expression of RanBPM and relation to S100A7 expression by in-situ hybridization and RT-PCR in a panel of pre-invasive and invasive breast tumors.

AIM 1.
To study the psoriasin –RanBPM interaction, I had cloned the RanBPM cDNA sequence into several expression vectors which would generate epitope tagged fusion proteins. This strategy was used because there are several antibodies commercially available to the various tags, where none existed for RanBPM. These constructs included one which generated a fusion protein of RanBPM with a C-terminal FLAG epitope tag, another which created a protein containing a N-terminal His and Xpress tag. Both vectors contained a mammalian promoter for high levels of transcription invivo. Unfortunately, many attempts to achieve RanBPM expression using these constructs transfected into several types of cultured cells failed.
The construct containing the N-terminal His and Xpress tags has a T7 promoter allowing for transcription and translation in vitro. Using this alternative in-vitro strategy we were able to generate RanBPM protein and to demonstrate that the interaction (first identified by yeast 2-hybrid assay) could occur in-vitro.

AIM2.
We next generated a new expression construct using a tetracycline inducible plasmid system in order to express untagged but inducible RanBPM. To detect the expected RanBPM protein we also tried to make a ployclonal antibody against RanBPM using the epitope described by Nakamura and colleagues (J Cell Biol 1998 Nov 16;143(4):1041-

52). Unfortunately, the antibody did not detect either transfected, invitro generated or endogenous RanBPM. We think this is due to the poor antigenicity of the epitope as many non-specific bands were detected. This finding was also confirmed soon after our own attempt in another series of experiments by the group that originally described RanBPM (Nishitani H et. al, Gene 2001 Jul 11;272(1-2):25-33). For all constructs, detection of mRNA produced by the transfected construct was examined and confirmed by RT-PCR. Using vector specific primers, we were able to detect mRNA produced from the transgene, although at low levels which may be due to low transfection efficiency. Recently, a report was published that found the RanBPM protein was larger than originally described on the basis of the new finding of a 5' sequence that was presumed to be a non-coding regulatory region of the gene in fact contains coding sequence. All of our expression constructs do not contain the new N-terminal piece and therefore may not represent the true RanBPM cDNA.

AIM 3.
We have examined RanBPM mRNA expression in a series of cell lines and breast tumors initially by Real-time RT-PCR before attempting in-situ hybridization. This assay is not dependant on the change in the size of the open reading frame or translated protein as our primers also detect the longer RanBPM. In the cell lines, expression was significantly higher in cells derived from tumors compared to cells representative of normal epithelia and fibroblasts (see manuscript in Appendix Fig3). In a selected series of 80 tumors, higher RanBPM expression was seen in tumors that were Estrogen Receptor positive and had lower expression of psoriasin. Expression of psoriasin in the tumor series was correlated with increased levels of inflammation (see manuscript in Appendix Table1), so we examined RanBPM mRNA expression in human peripheral blood mononuclear lymphocytes. It was found that the polyclonal activator PHA which elicits dominantly Th2 associated cytokine response induced RanBPM mRNA production compared to polyclonal activators that elicit Th1 dominated immunity (anti-CD3, TSST-1) or cytokine production by antigen presenting cells that is Th1 inducing (LPS, SAC). No significant differences were apparent in RanBPM expression between male (n=3) and female (n=2) subjects in this sample.

Summary
We have therefore completed most of AIMS 1 and 3, and initiated experiments for AIM 2. However we have run into roadblocks with our studies of RanBPM (failure to develop an antibody or achieve in-vivo expression with the originally described cDNA) and learnt that the originally described cDNA that we had focused our studies on, is not the full length RanBPM. More importantly the full length RanBPM was not shown to have the same biological effects as originally described and therefore should now be regarded as a gene of unknown function (although a very recent paper suggests it may be involved in signal transduction). Our studies on psoriasin's interaction with RanBPM are described in the publication in Appendices page 14-20.

While we are considering this information about RanBPM, and the uncertainty as to its function, we went back to review the results of our 2-hybrid screen for proteins interacting with psoriasin and from which RanBPM was isolated. We found that much

more was now known about the identity and function of another interacting protein, Jab1 (c-jun activation domain-binding protein-1) that we had also identified. We asked for and received approval to modify the Statement of Work to pursue the interaction of psoriasin with Jab1.

We became very interested in pursuing the psoriasin-Jab1 interaction further as outlined in the below accepted revision of the Statement of Work.

AIM 1.

12-18   Co-localize Jab1 and psoriasin in breast cells and study biochemical interaction co-immunoprecipitation.

18-24   Determine if psoriasin has effects on functions of Jab1 such as AP-1 activity and p27 degradation in psoriasin transfected breast cells.

AIM 2.

24-36   Define psoriasin's structural binding site for Jab1 by site directed mutagenesis to prove interaction.

AIM 3.

24-36   Examine cell type, localization and level of expression of Jab1 in relation to psoriasin expression by immunohistochemistry in breast tumors to correlate Jab1's location and expression in relation to psoriasin.

AIM 1.

See attached publication in Appendices section, page 21-28.

AIM 2.

Psoriasin was identified to contain a putative Jab1 binding motif in its C-terminal portion. This domain was mutated by site directed mutagenesis. MDA-MB-231 breast cancer cells stably expressing the 'mutant' psoriasin were generated and characterized for their ability to interact with Jab1 and influence Jab1 activity. Immunoprecipitation studies with Jab1 showed a decrease in the affinity of the interaction for 'mutant psoriasin' compared to unmodified psoriasin (Appendices, Fig 1). This decrease in ability to form a stable interaction was recapitulated by the observation that 'mutant psoriasin' did not have a

noticeable effect on p27 degradation compared to non expressing parentals and controls. A similar observation was seen for phospho c-jun as well (Appendices, Fig 2).

AIM 3.

See attached submitted manuscript in Appendices section, page 29-51.

Key Research Accomplishments

- Psoriasin interacts with Jab1 *in vivo*

- Psoriasin alters Jab1 functions such as increasing AP-1 activity and increasing p27 degradation.

- Mutation of the putative Jab1-binding domain in psoriasin results in a decreased interaction with Jab1 and a decrease in Jab1 activity.

- Psoriasin found to be associated with poorer patient outcome and recurrence in Estrogen Receptor negative invasive breast tumors.

Reportable Outcomes

Manuscripts Published:

1) Emberley ED, Niu Y, Njue C, Kliewer EV, Murphy LC, Watson PH.
Psoriasin (S100A7) expression is associated with poor outcome in estrogen receptor-negative invasive breast cancer.
Clin Cancer Res. 2003 Jul;9(7):2627-31.

2) Emberley ED, Niu Y, Leygue E, Tomes L, Gietz RD, Murphy LC, Watson PH.
Psoriasin interacts with Jab1 and influences breast cancer progression.
Cancer Res. 2003 Apr 15;63(8):1954-61.

3) Alowami S, Qing G, Emberley E, Snell L, Watson PH.
Psoriasin (S100A7) expression is altered during skin tumorigenesis.
BMC Dermatol. 2003 Feb 24;3(1):1.

4) Emberley ED, Gietz RD, Campbell JD, HayGlass KT, Murphy LC, Watson PH.
RanBPM interacts with psoriasin in vitro and their expression correlates with specific clinical features in vivo in breast cancer.
BMC Cancer. 2002 Nov 6;2(1):28.

Manuscripts submitted:

Ethan D. Emberley, Salem Alowami, Linda Snell, Leigh C Murphy, and Peter H. Watson
S100A7 (Psoriasin) expression is associated with aggressive features in Ductal Carcinoma In-Situ (DCIS) of the Breast

Abstracts, Year 3:

1) The third Era of Hope meeting for the Department of Defense (DOD) Breast Cancer Research Program (BCRP)- Sept 2002
PSORIASIN (S100A7) INFLUENCES MALIGNANT PROGRESSION IN BREAST CANCER.
Ethan D. Emberley, Yulian Niu, A. Kate Hole, R. Daniel Gietz, Leigh C. Murphy, and Peter H. Watson

2) University of Manitoba Research Day- May 2003
THE PSORIASIN GENE AND BREAST CANCER PROGRESSION.
Ethan Emberley, Yulian Niu, R. Daniel Gietz, Leigh C. Murphy, and Peter H. Watson

3) American Association for Cancer Research Annual Meeting- July 2003
PSORIASIN (S100A7) EXPRESSION IS ASSOCIATED WITH POOR OUTCOME IN
ESTROGEN RECEPTOR NEGATIVE INVASIVE BREAST CANCER.
Ethan D. Emberley, Yulian Niu, Catherine Njue, Erich V. Kliewer, Leigh C. Murphy,
Peter H. Watson.

Abstracts for total term of Award:

Poster Presentation 2001 American Association for Cancer Research Annual Meeting.
DIFFERENTIAL GENE EXPRESSION ANALYSIS OF MICRODISSECTED DUCTAL
CARCINOMA IN SITU (DCIS) OF THE BREAST. Adewale Adeyinka, Ethan D.
Emberley, Charles C. Wykoff, Adrian L. Harris, Leigh C. Murphy, Peter H. Watson

Oral Presentation 2001 Canadian Breast Cancer Research Initiative.
THE DIFFERENTIALLY EXPRESSED PSORIASIN GENE: POTENTIAL ROLE IN
BREAST CANCER PROGRESSION. Ethan D. Emberley, A. Kate Hole, R. Daniel Gietz,
Leigh C. Murphy, Peter H. Watson.

Oral Presentation 2001 Canadian Breast Cancer Research Initiative.
PRE-INVASIVE BREAST CANCER (DCIS) ASSOCIATED WITH HIGH-RISK AND
LOW-RISK OF RECURRENCE DIFFER IN THEIR PATTERNS OF GENE
EXPRESSION. Adewale Adeyinka, Ethan D. Emberley, Charles C. Wykoff, Adrian L.
Harris, Leigh C. Murphy, Peter H. Watson.

Poster Presentation 2001 Canadian Breast Cancer Research Initiative.
HYPOXIA ASSOCIATED GENE EXPRESSION IN BREAST CANCER. Ladislav
Tomes, Ethan Emberley, Adewale Adeyinka, Peter Watson.

Poster Presentation at 2001 University of Manitoba Research Day.
A DIFFEREMTIALLY EXPRESSED BREAST CANCER GENE AND ITS
INTERACTION WITH CENTROSOMAL PROTEINS. Ethan D. Emberley, A. Kate Hole,
R. Daniel Gietz, Leigh C. Murphy, Peter H. Watson.

Poster Presentation at 2002 Oncogenomics Research Conference.
PSORIASIN (S100A7) INFLUENCES MALIGNANT PROGRESSION IN BREAST
CANCER. Ethan Emberley, Yulian Niu, Leigh Murphy, Peter Watson.

Poster Presentation at 2002 University of Manitoba Research Day.
PSORIASIN (S100A7) INFLUENCES PROGRESSION IN BREAST CANCER. Ethan D.
Emberley, Yulian Niu, Ladislav Tomes, Catherine Njue, Erich V. Kliewer, Etienne
Leygue, R. Daniel Gietz, Leigh C. Murphy, Peter H. Watson.

<u>Conclusion</u>

We have shown that psoriasin can contribute to breast tumor progression and that its action may be mediated, at least in part, through Jab1. Although other important cellular proteins also influence and may compete for Jab1, psoriasin is one of the most abundant proteins in high-risk DCIS and is likely to exert an important effect on Jab1 activity in breast tumor cells at an early stage of tumor progression. In breast tumors we found that psoriasin expression on its own may be an independent prognostic factor for outcome in invasive breast cancer. It will be important to explore the prognostic significance of psoriasin in preinvasive DCIS and as an indicator of risk of progression to invasive disease.

Appendices

Figure 1: Co-immunoprecipitation of Jab1 with mutated and unmodified psoriasin. 231 parental cell line does not express psoriasin. 231-HP2 is a clone expressing high levels of psoriasin. Jab1 antibody was omitted in the 231-HP2 –Ab lane, hence no detectable Jab1 protein. PTM 231.14 is a clone expressing high levels of our mutated psoriasin. No Jab1 is seen in the PTM 231.14 –Ab lane as Jab1 antibody was omitted. MDA-MD-468 is a breast cancer cell line expressing high levels of psoriasin and was used as a positive control.
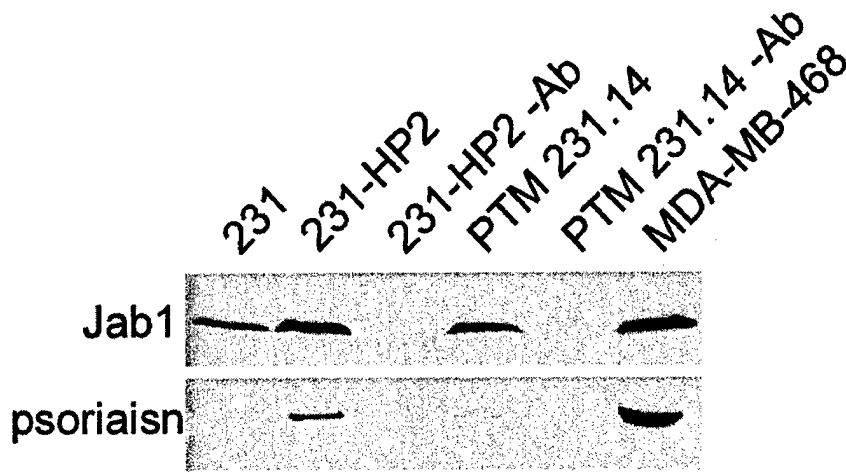
Figure 2: Western blot showing increased levels of c-jun in the presence of unmodified psoriasin (231-HP1 and 231-HP2) but no increase in the presence of mutated psoriasin (231 PTM.2 and 231 PTM.14). p27 levels drop in the presence of unmodified psoriasin but mutated psoriasin does not appear to affect p27 degradation. Actin was used to show equal loading of total protein.
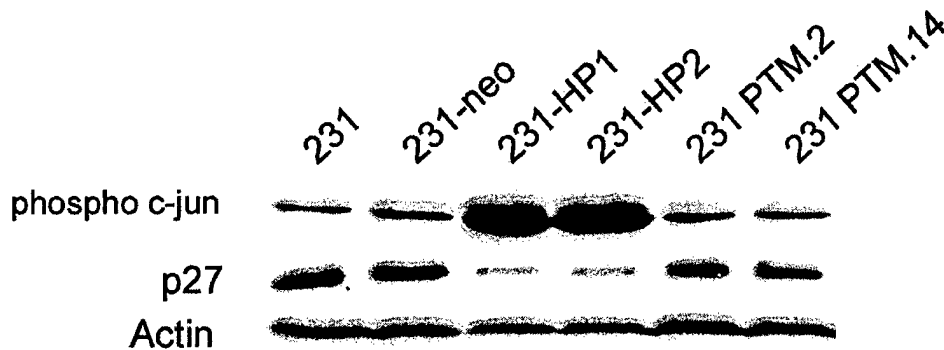
13

Research article

RanBPM interacts with psoriasin in vitro and their expression correlates with specific clinical features in vivo in breast cancer

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Abstract

Background: Psoriasin has been identified as a gene that is highly expressed in pre-invasive breast cancer, but is often downregulated with breast cancer progression. It is currently unknown whether psoriasin influences epithelial cell malignancy directly or by affecting the surrounding environment. However the protein is found in the nucleus, cytoplasm as well as extracellularly. In the present study we have sought to identify potential psoriasin-binding proteins and to describe their expression profile in breast tumors.

Methods: The yeast two-hybrid method was used to identify potential binding partners for psoriasin. The interaction of psoriasin with RanBPM was confirmed in-vitro by co-immunoprecipitation. The expression of RanBPM and psoriasin was measured by RT-PCR in a series of breast cell lines, breast tumors and primary lymphocytes.

Results: We have identified RanBPM as an interacting protein by the yeast two-hybrid assay and confirmed this interaction in-vitro by co-immunoprecipitation. RT-PCR analysis of RanBPM mRNA expression in cell lines (n = 13) shows that RanBPM is widely expressed in different cell types and that expression is higher in tumor than in normal breast epithelial cell lines. RanBPM expression can also be induced in peripheral blood mononuclear cells by treatment with PHA. RanBPM mRNA is also frequently expressed in invasive breast carcinomas (n = 64) and a higher psoriasin/RanBPM ratio is associated with both ER negative ($p < 0.0001$) and PR negative status ($p < 0.001$), and inflammatory cell infiltrates ($p < 0.0001$) within the tumor.

Conclusions: These findings support the hypothesis that psoriasin may interact with RanBPM and this may influence both epithelial and stromal cells and thus contribute to breast tumor progression.

Background

We have previously identified the psoriasin gene (S100A7) as highly expressed in pre-invasive breast cancer and subsequently found that persistent psoriasin expression in invasive breast cancer is associated with markers of poor prognosis [1]. The S100 proteins are calcium-binding proteins that may influence several cellular activities such as growth, chemotaxis, adhesion and cytoskeletal activity [1–3]. The function of psoriasin and its influence in breast cancer is unknown. However *in vitro* and *in vivo* study of its role in psoriasis [4] and also in breast tumors [1] suggests that one possible role may be to influence the inflammatory response as a secreted chemotactic factor. We [1] and others [5] have also observed nuclear and cytoplasmic expression within squamous and breast epithelial cells which raises the possibility of an additional function within epithelial cells. We therefore sought to pursue a possible intracellular function by utilizing the yeast 2-hybrid approach to screen a normal human breast expression library for intracellular breast epithelial proteins that might interact with psoriasin and that might offer insights into its functional role in breast tumor progression.

Methods

Yeast two-hybrid library screen

The full-length psoriasin coding sequence was cloned in frame with the Gal4 DNA Binding Domain (BD) of the Bait plasmid pGBT9 (Clontech) by PCR. pGBT9-psor was transformed into *Saccharomyces cerevisiae* strain KGY37 (supplied by R.D. Gietz) followed by growth on selective media. Expression of the psoriasin fusion protein was confirmed by Western Blot (data not shown). A normal human mammary gland cDNA Prey library (Clontech) was screened for proteins that potentially interact with psoriasin. We screened 1.73×10^7 clones (4X the complexity of the library) and 242 colonies were picked to assay for expression of the LacZ reporter gene. Clones showing positive LacZ expression had the library plasmid isolated to test for the specificity of their interaction with psoriasin.

Immunoprecipitation

Full-length psoriasin coding sequence cloned into pcDNA3.1 (InVitrogen), and RanBPM (amino acids 230–730) cloned into pcDNA4 HisMax (InVitrogen) in-frame with the N-terminal His-Xpress epitope tag. ^{35}S-Met labelled protein was generated using the Wheat Germ TNT kit for psoriasin and Rabbit Reticulocyte TNT kit for RanBPM according to manufacture's protocol (Promega). Labeled proteins were co-immunoprecipitated in buffer (50 mM Tris-HCl pH 7.5, 137 mM NaCl, 0.05 mM CaCl$_2$, 0.05% Triton X-100) using anti-HisG antibody (InVitrogen). Samples were electrophoresed through a 15% Tris-Tricine polyacrylamide gel as previously described [1].

The gel was then dried and autoradiography to detect labeled proteins.

Cell lines and tumor tissue samples

Peripheral blood leucocytes were obtained from five healthy subjects by venepuncture and cultured as previously described [6] Breast cancer cell lines were obtained from the ATCC and were cultured as previously described [1] in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin and L-Glutamine. MCF10A cells were the kind gift of Dr. Fred Miller (Wayne State University, Detroit MI). All cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO$_2$. Breast tumor cases were selected from the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada), which operates with ethical approval from the University of Manitoba's Ethics Board and processed previously described [7]. Tumors with estrogen and progesterone receptor levels above 3 fmol/mg total protein and 15 fmol/mg total protein, respectively, were considered ER or PR positive.

The study cohort comprised 64 invasive carcinomas selected to represent a range of tumor types and on the basis of high tissue quality, presence of invasive tumor within >35% of the cross section of the frozen block and minimal (<5%) normal or *in situ* epithelial components. The study group included different invasive tumor types (34 ductal, 13 lobular, and 17 'special type' tumors), and a range of ER status (19 negative, 45 positive), PR status (30 negative, 34 positive) and stages (37 node negative, 27 node positive).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

RT-PCR was performed on extracted total RNA (200 ng) that was reverse transcribed as described previously [8]. Real-time Quantitative PCR and analysis was performed with the Roche LightCycler. Each PCR reaction was performed in a total volume of 20 µl and consisted of 0.2 µl of each sense and anti-sense primer at 50 µM, 1.6 µl of 25 µM MgCl$_2$, 15 µl H$_2$O, 2 µl Master Mix (LightCycler- DNA Master SYBR Green I) and 1 µl cDNA template.

The psoriasin primers were sense (5'-AAG AAA GAT GAG CAA CAC-3') and antisense (5'-CCA GCA AGG ACA GAA ACT-3') corresponding to the cDNA sequence. RanBPM primers were sense (5'-CGC ACA TTT TTC AGG TTT-3') and antisense (5'-CTT GCC ACA GTC TCT CCT T-3'). Cyclophilin 33 was used as a loading control for analysis of RanBPM in cell lines and PBMCs using sense (5'-GCT GCG TTC ATT CCT TTT G-3') and antisense (5'-CTC CTG GGT CTC TGC TTT G-3'). All PCR primers were designed to span at least one intron-exon boundary to prevent amplification of possible genomic DNA contamination. For
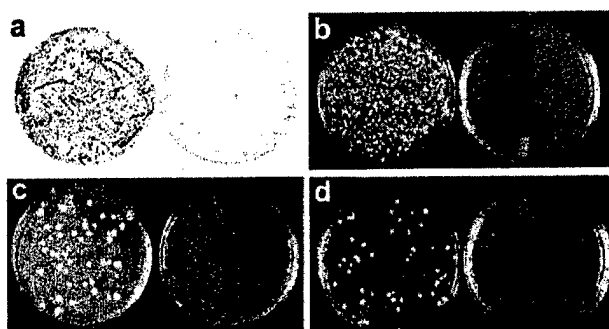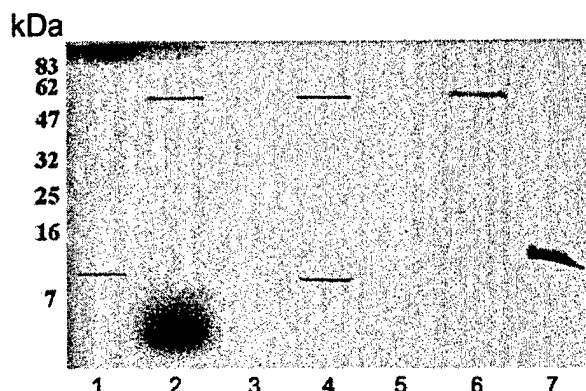
Figure 1
Confirmation of specificity of interactions observed in yeast 2-hybrid assay. Panels show yeast transformed with a) psoriasin and RanBPM expression plasmids (left) and RanBPM alone (right), and yeast transformed with b) Psoriasin alone, c) psoriasin and empty prey vector, and d) psoriasin with a control gene not isolated in the primary screen. In panels b-d, plates on left are Histidine + (control) and plates on the right are Histidine − (test). Panel a) shows activation of LacZ reporter gene only occurs in yeast transformed with both expression plasmids (left) and no activation in yeast transformed with RanBPM alone (right). Panel b) shows that psoriasin alone cannot activate the Histidine reporter gene as demonstrated by absence of growth on Histidine − plate. Panels c) and d) show that there is a specific interaction necessary for activation of the reporter gene.

Figure 2
In vitro interaction of psoriasin and RanBPM as determined by co-immunoprecipitation. ^{35}S-Met labelled proteins were generated. Psoriasin (lane 1) and RanBPM (lane 2) were electrophoresed through a denaturing polyacrylamide gel and detected by autoradiography. Psoriasin binds to and co-immunoprecipitates with RanBPM (lane 4). RanBPM and psoriasin do not bind to protein G-beads on their own, (lane 3 and 5 respectively). RanBPM does not interact with mouse submaxillary gland protein when either RanBPM (lane 6) or mouse submaxillary gland protein (lane 7) is precipitated.

PCR amplification in the LightCycler melting curve analysis was preformed at the end of the cycling program to confirm specificity of amplification. Psoriasin, cyclophilin 33 and RanBPM PCR products were each cloned into pGEM-T Easy (Promega), sequenced and then serially diluted separately to construct the standard curve used for quantification of mRNA expression.

Tumors from each cohort were processed as a batch, from frozen sectioning to RNA extraction, reverse transcription in duplicate and then duplicate PCRs from each RT reaction. Controls included RT- and RNA- controls. RNA from PBMLs of five subjects were collected from 3×10^6 cells after 24-hour treatment (10 µg/ml PHA, 0.1 ng/ml TSST-1, 30 ng/ml anti-CD3, 10 ng/ml LPS and SAC stimulation at 0.0025% and 0.00025%) as well as unstimulated cells grown for 24 hours in media alone. All RNA isolations were performed using Tri-reagent according to the manufacture's directions (Sigma).

Statistical analysis
For statistical analysis, psoriasin and RanBPM mRNA levels and the psoriasin/RanBPM ratio were assessed either as a continuous variable or transformed into low or high expression categories using the 75th percentile as a cutpoint for all variables. This cutpoint was selected as it corresponded to the minimum mRNA level at which psoriasin

protein could be detected by Western blot analysis of frozen sections from a subset of the tumors (Data not shown). Correlations were tested using Spearman's test and associations with categorical variables were tested by Chi-squared test. For induction of RanBPM in PBMC, one-way ANOVA (nonparametric) analysis was performed on the means of duplicate Reverse Transcription samples for selected treatments.

Results
Yeast two-hybrid screen and identification of true positives
The yeast Bait vector pGBT9-psoriasin was used to screen a normal human mammary gland cDNA expression library (Clontech) to identify potential proteins with which psoriasin forms a physical interaction. Clones that grew on selection media were isolated and examined for the strength and specificity of their interaction with psoriasin. Four clones were identified that specifically interacted with psoriasin in the yeast system. We pursued the interaction of RanBPM (clone 6-3) with psoriasin as it presented the strongest interaction, as determined by the intensity of activation of the reporter gene LacZ (data not shown). Psoriasin was demonstrated to specifically interact with RanBPM in the yeast system (Figure 1).

Co-immunoprecipitation of psoriasin and RanBPM
Both psoriasin and RanBPM were cloned by PCR into their respective expression vectors downstream of a T7 promoter and ^{35}S-Met labeled proteins were generated.
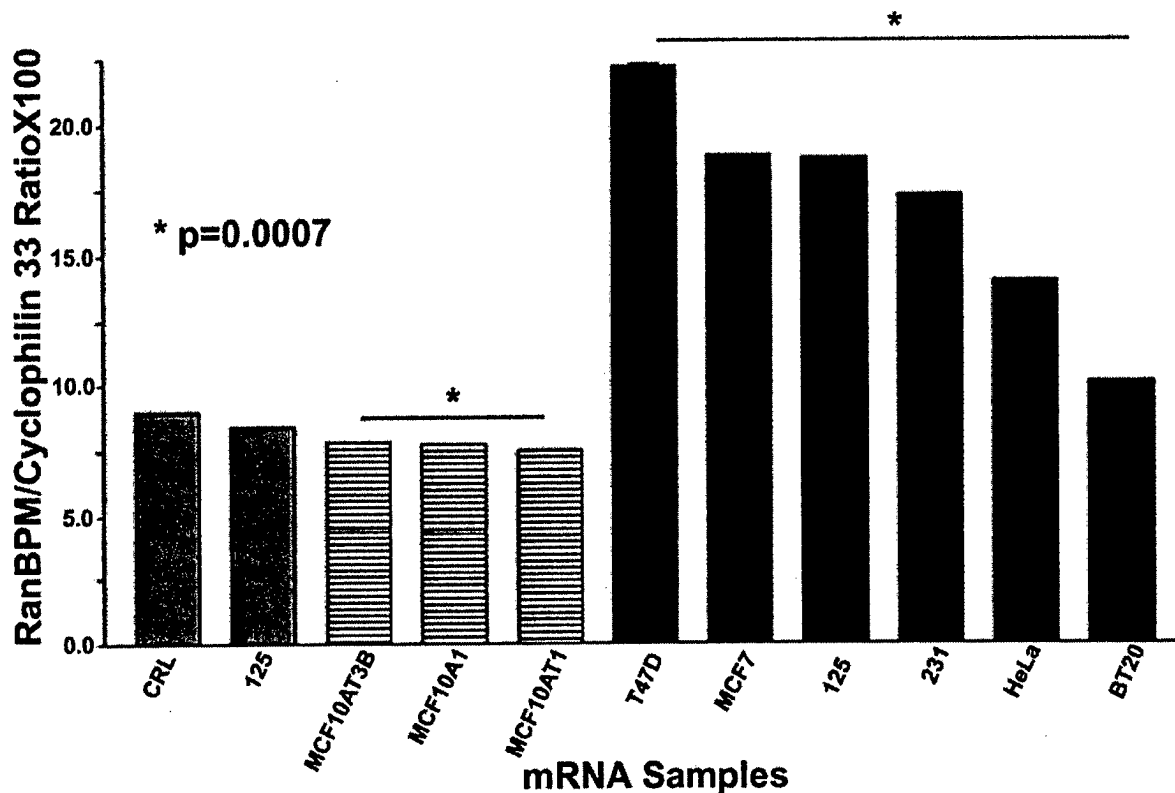
Figure 3
RanBPM mRNA expression in selected cell lines. RanBPM mRNA expression in fibroblasts (CRL and 125), normal mammary epithelia (MCFI0ATB, MCFI0AI, MCFI0ATI), and epithelial carcinoma cell lines from breast (T47D5, MCF-7, 126, MDA-MB-231, BT20) and cervix (HeLa). RanBPM is expressed at higher levels in cells derived from tumors compared to those derived from normal epithelia and stroma. Columns and bars represent means and standard deviations from duplicate experiments. Mann Whitney test was used to compare levels between normal epithelial cells and fibroblasts (*) or neoplastic epithelial cells (**).

The radiolabelled proteins were immunoprecipitated by addition of anti-HisG antibody, which specifically detects the His-tagged RanBPM protein. As shown (Figure 2), the protein-protein interaction observed in yeast was confirmed by these co-immunoprecipitation studies.

Cell line expression

RanBPM mRNA expression was assessed by RT-PCR in established fibroblast and epithelial cell lines and in freshly obtained peripheral blood mononuclear cells (Figure 3). RanBPM was detected in all cells and the relative levels differed between cell types. Amongst the established cell lines there was no significant difference between fibroblasts, normal epithelial cells and Ras-transformed derivatives of the same normal epithelial cell. RanBPM expression was consistently higher in tumor cell lines compared to normal cells (p = 0.0007), with up to a 3-fold difference in levels. Within the set of 5 breast tumor cell lines examined, no relationship with ER status or epithe-

lial differentiation was evident. Psoriasin expression was undetectable or very low in all cell lines (data not shown).

RanBPM expression was also examined in primary cultures of peripheral blood mononuclear cells. In experiments with samples from 5 healthy subjects, RanBPM mRNA was strongly induced in cells following 24-hour stimulation with the polyclonal activator PHA (p < 0.001). PHA elicits dominantly Th2 associated cytokine response [6]. In contrast, cells stimulated by polyclonal activators that elicit Th1 dominated immunity (anti-CD3, TSST-1) or cytokine production by antigen presenting cells that is Th1 inducing (LPS, SAC), an increase in RanBPM expression was weak to undetectable (Figure 4). No significant differences were apparent in RanBPM expression between male (n = 3) and female (n = 2) subjects in this sample.
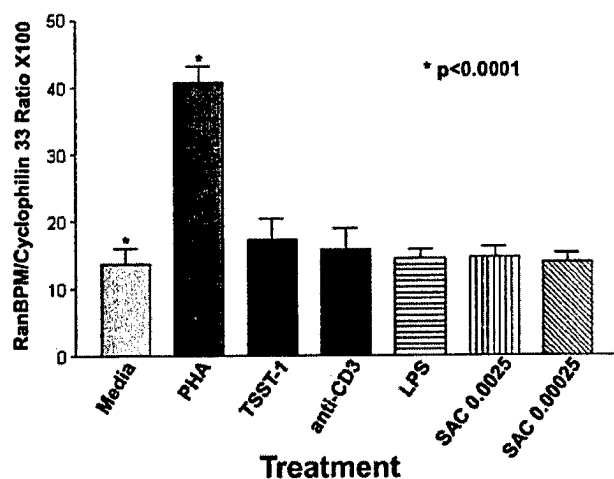
Figure 4
RanBPM mRNA expression in peripheral blood mononuclear cells (PBMCs). RanBPM mRNA expression is strongly induced by stimulation of cells with PHA, weakly induced by TSST-1, but not anti-CD3, LPS or SAC. Columns and bars represent means and standard deviations from duplicate experiments performed on samples from 5 donors.

Tumor expression

The relationship between RanBPM, psoriasin, the psoriasin/RanBPM ratio and indicators of differentiation and prognosis was examined in a series of 64 invasive breast tumors by quantitative RT-PCR (Table 1). Higher levels of expression of either RanBPM or psoriasin were present in only 16/64 and 16/64 tumors respectively, however high levels of expression of both genes was observed to coincide in 4/64 cases. This small subset of cases showed no significant distinguishing clinical features. Higher RanB-PM mRNA levels were seen in association with ER positive status and low inflammation. However there was no significant difference in RanBPM mRNA expression with any parameter including indicators of differentiation (ER, PR, tumor type or grade) or stage (tumor size and nodal status) or with cellular composition of the tumor section (inflammation and proportion of epithelial tumor cells and stroma). At the same time higher levels of psoriasin expression were significantly associated with ER negative and PR negative status and inflammation (Table 1). The psoriasin/RanBPM ratio showed even stronger and highly significant associations with the same three parameters, and it was also higher in medullary carcinomas compared to other special type tumors (mucinous and tubular), lobular, and ductal carcinomas (p = 0.0313).

Discussion

To pursue the role of psoriasin (S100A7) expression in breast cancer we sought interacting proteins in breast epithelial cells using the yeast 2-hybrid assay. We have iden-

tified RanBPM as a potential interacting protein and confirmed this interaction by *in vitro* assay. Study of cell lines and breast tumors shows that RanBPM mRNA is widely expressed and that while RanBPM expression shows no specific relationship with markers of differentiation or prognosis, high levels of expression of both genes is present in some tumors, and a strong association exists between the psoriasin/RanBPM ratio and both ER/PR status and inflammatory cell infiltrates within the tumor.

The biological role of psoriasin in breast tumors is not known, however it may be related to aspects of tumor progression. This role might be mediated either through an indirect influence on the host immune response or through a more direct influence on the epithelial tumor cell. The first hypothesis is supported by correlations between psoriasin and the intensity of the host inflammatory cell response within invasive breast tumors [1] and *in vitro* effects as a secreted chemotactic factor for T-cells [4]. The second hypothesis is supported by evidence that psoriasin may not only be secreted [9,10]. but also can be localized in both nuclear and cytoplasmic epithelial cell compartments in normal skin and breast tumors [1]. Other secreted S100 proteins have also been localized to cytoplasm and nucleus [11] and altered subcellular localization in disease has also been observed with a keratinocyte S100 related protein, profilaggrin [12,13].

RanBPM was originally described as a Ran binding protein that is highly conserved between human, mouse and hamster, and detectable in both skin fibroblast and HeLa cell lines [14]. However, more recently it has been found that the original study had centered on a N-terminally truncated cDNA and that the full size RanBPM cDNA encodes a larger 90 kDa protein that localizes to both the nucleus and also to the peri-nuclear and peri-centrosomal cytoplasmic region in HeLa cells [15]. While RanBPM was identified through its ability to interact with the Ran nuclear-cytoplasmic transport protein in the yeast system, when overexpressed the c-terminal portion of RanBPM was capable of causing reorganization of the microtubule network and ectopic nucleation of microtubules *in vivo*, and this effect could also be inhibited by GTP-Ran [14]. This is of interest as although Ran is known to play a key role in nuclear transport [16,17], it has also been associated with a variety of additional functions, including cell proliferation and viability in breast cells [18] that may be mediated by interactions with a number of Ran binding proteins. While the full length RanBPM has now been identified, the exact role for this protein in microtubule function remains to be explored. Our findings here confirm that RanBPM is expressed in multiple cell types and show that it may be increased in breast tumor cell lines. In breast tumors higher levels of expression of RanBPM and psoriasin overlapped in some cases. While this small sub-

Table 1: Relationship Between RanBPM and Psoriasin Expression and Clinical-Pathological Parameters in Human Breast Cancer

		Ran BPM			Psoriasin			Psoriasin/RanBPM		
		low	high	p value	low	high	p value	low	high	p value
ER	-ve	16	3	ns	8	11	0.0002	7	12	<0.0001
	+ve	32	13		40	5		41	4	
PR	-ve	23	7	ns	17	13	0.001	17	13	0.002
	+ve	25	9		35	3		31	3	
INFL	low	36	15	ns	41	10	0.049	44	7	<0.0001
	high	12	1		15	6		4	9	
NS	neg	28	9	ns	30	7	ns	27	10	ns
	pos	20	7		18	9		21	6	
GRADE	low	14	3	ns	15	2	ns	15	4	ns
	int	13	5		12	6		13	5	
	high	9	3		9	3		8	4	
SIZE	< = 2	11	5	ns	10	3	ns	11	5	ns
	>2	27	12		32	11		32	7	
TYPE	ductal	26	9		26	9		25	9	
	lobular	11	2		11	2		11	2	
	mucinous	3	3		4	1		5	1	
	tubular	4	2		4	2		5	1	
	medullary	5	0		3	2		1	4	

set of cases showed no significant distinguishing clinical features and there was no association with known prognostic factors, the expression of RanBPM was inversely related to psoriasin. There was also a tendency to higher levels of RanBPM in association with ER positivity and low levels of inflammation, leading to highly significant differences in the psoriasin/RanBPM ratio in tumors that differed by ER status and extent of inflammation. This latter association is also supported by the higher psoriasin/RanBPM ratio found in medullary carcinomas compared to other invasive tumors, where higher levels of inflammation are inherent in the diagnosis of this special tumor type. Our data also suggests that RanBPM is rapidly expressed by primary PBMC, in particular upon stimulation leading to type-2 immunity. This raises the possibility that RanBPM could be a target for mediating the actions of se-

creted psoriasin, perhaps affecting T cell trafficking to activated or inflamed sites.

Psoriasin has also been found to interact with two other proteins. It is a substrate for transglutaminases TG1 and TG2 [19], and a binding partner for epidermal fatty acid binding protein (E-FABP) in epidermis [5]. Transglutaminases are expressed in both epithelium and inflammatory cells and their cross linking capacity is associated with a range of functions including apoptosis and terminal differentiation in keratinocytes, cross linking of extracellular matrix proteins, and interaction with integrins to mediate adhesion and motility of monocytic cells [20]. The FABP proteins have also been implicated in terminal differentiation in both epidermis and breast epithelium where overexpression of H-FABP and B-FABP can both cause

growth inhibition and reduction of tumorigencity in mammary carcinoma cells. In the CNS, B-FABP has also been implicated in the control of the glial fibre system and neuronal migration. RanBPM is a third interacting protein, that is expressed in several cell types, is induced in PHA stimulated peripheral blood leucocytes, and when overexpressed as a truncated protein can affect microtubule function. The interaction with psoriasin may just reflect a common mode of intracellular transport. However, one common functional association to these three interacting proteins is an involvement in cytoskeletal functions, adhesion, and migration. Alterations of adhesion and polarity are associated with the progression of breast cancer and are inherent in the process of invasion. While psoriasin expression in keratinocytes [21] is associated with changes in adhesion, it remains to be determined if manipulation of psoriasin in squamous and breast epithelial cells can directly influence cell adhesion.

In conclusion, we have shown that psoriasin interacts with an intracellular protein, RanBPM in-vitro. Further study to confirm the interaction in-vivo and explore the biological role of RanBPM in breast and inflammatory cells may help to elucidate the role of psoriasin in breast cancer.

Competing interests
None declared

Authors' contributions
EE performed the Yeast 2-hybrid screen, co-immunopreciputation studies, RT-PCR analysis. RDG assisted in the Yeast 2-hybrid screen. JDC cultured and stimulated the PBMC cultures. KTH designed the PBMC experiments. LCM participated in the design of the study. PHW conceived of the study, and participated in its design and coordination.

Acknowledgements

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Psoriasin Interacts with Jab1 and Influences Breast Cancer Progression[1]

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ABSTRACT

Psoriasin (S100A7) is expressed at low levels in normal breast epithelial cells but is highly expressed in preinvasive ductal carcinoma *in situ*. Persistent psoriasin expression occurs in some invasive carcinomas and is associated with poor prognostic factors. Whereas there is evidence that secreted psoriasin can act as a chemotactic factor for CD-4-positive lymphocytes in psoriatic skin lesions, an intracellular biological function is unknown. We have found that psoriasin physically interacts with Jab1 (c-jun activation-domain binding protein 1) in the yeast two-hybrid assay and confirmed this by coimmunoprecipitation assay in breast cancer cells. Psoriasin-transfected breast cancer cells showed increased nuclear Jab1 and demonstrated several features consistent with an alteration in Jab1 activity including an increase in activator protein-1 (AP-1) activity, increased expression of AP-1 and *HIF-1*-dependent genes, and reduced expression of the cell-cycle inhibitor p27^{Kip1}. Psoriasin overexpression was also associated with alteration of cellular functions that are associated with increased malignancy, including increased growth, decreased adhesion, and increased invasiveness *in vitro*, as well as increased tumorigenicity *in vivo* in nude mice. We conclude that intracellular psoriasin influences breast cancer progression and that this may occur through stimulation of Jab1 activity.

INTRODUCTION

We have identified psoriasin (S100A7) previously as a differentially expressed gene between DCIS[3] and invasive carcinoma (1). The expression of psoriasin is low in normal breast and benign pathologies (1), but psoriasin is among the most highly expressed genes in high grade DCIS (2, 3). Whereas expression is often reduced in invasive carcinoma, persistent high expression is associated with markers of poor prognosis (4). This profile of gene expression raises the possibility that psoriasin may be functionally involved in invasion and early tumor progression (5). Psoriasin is a small calcium-binding protein belonging to the S100 gene family (6, 7), among which several other members have been associated with breast tumor progression (8, 9). Most interest has been focused on *S100A4* (10), which was also initially identified as a differentially expressed gene between non-metastatic and metastatic rodent mammary tumor cell lines (11). In later studies by several groups, *S100A4* has been shown to directly influence the invasive and metastatic phenotype in breast cancer cell

lines (12–14) and tumors (15), and expression is also associated with poor prognostic factors and patient survival in human breast tumors (16).

Psoriasin was originally described as highly expressed within psoriatic skin lesions (17) and found to be a secreted protein (18), but has since been observed to be present in the cytoplasm and nucleus of both abnormally differentiated keratinocytes (19) and breast carcinoma cells (2, 4). Whereas there is evidence that secreted psoriasin can act as a chemotactic factor for CD-4-positive lymphocytes in psoriatic skin lesions (18), a function for intracellular psoriasin also appears likely but has yet to be established.

We sought to identify proteins that might interact with psoriasin in breast epithelia by using the yeast two-hybrid system (20). Jab1 (21) was found to specifically interact with psoriasin in the yeast system, and this interaction was confirmed by biochemical assay in breast cancer cells. Jab1 is a component of a multimeric protein complex (22, 23), the CSN/COP9 signalosome, which is involved in signal transduction and protein degradation via the Ub-26S proteasome (24, 25). The effect of overexpression of psoriasin on Jab1 distribution and function in a breast cancer cell line was studied. Psoriasin overexpression resulted in redistribution of Jab1 to the nucleus and multiple functional changes that can be attributed to activation of Jab1, as well as enhanced tumorigenesis and metastasis in an *in vivo* assay. These data support our hypothesis that psoriasin enhances early tumor progression and the process of invasion in breast cancer cells in part by interacting with Jab1 and positively enhancing its activity.

MATERIALS AND METHODS

Yeast Two-Hybrid System. For yeast two-hybrid studies, the coding region of human psoriasin protein was fused in-frame with the GAL4 DNA-binding domain of the pGBT9 vector (Clontech). The resulting bait plasmid (pGBT9-psor) was used to screen a normal human mammary epithelium cDNA library (Clontech) by the yeast two-hybrid method as we have described previously (26). Clones were isolated that could grow on Trp⁻ Leu⁻ His⁻ medium, did not autoactivate the β-galactosidase reporter gene, and demonstrated specificity for their interaction with psoriasin. This was done by testing the interaction of psoriasin with specific "prey" constructs not identified in the screen. Jab1 was analyzed in a similar fashion. The NH₂-terminal "bait" psoriasin plasmid was used to define the region of psoriasin involved in Jab1 binding encoded amino acids 1–52 (pGBT9-N-term-psor), and the COOH-terminal bait psoriasin plasmid encoded acids 43–101 (pGBT9-C-term-psor).

Cell Culture, Transfections, and Antibodies. The human breast carcinoma cell lines MDA-MB-231 and MDA-MB-468 were cultured in DMEM supplemented with 10% FBS under standard conditions (4). The former cell line is negative for psoriasin, whereas the latter expresses psoriasin mRNA and protein (confirmed by RT-PCR and Western blot; data not shown). The full psoriasin protein coding sequence was cloned into pcDNA3.1 (Invitrogen) and transfected into MDA-MB-231 cells using Superfect (Qiagen) followed by G418 selection. Resistant colonies were isolated and expanded. Psoriasin protein expression was determined by Western blot using a rabbit antipsoriasin antibody generated by our laboratory and directed against the epitope KQSH-GAAPCSGGSQ corresponding to amino acids 88–101. The specificity of the antibody was established by comparison with a similar antibody generated previously against the same epitope (4), and by immunohistochemistry and Western blot, using transfected breast cancer cell lines and tumors as described previously (4). Three MDA-MB-231 clones were found to express psoriasin

(designated as clone 231-LP1 exhibiting low psoriasin expression, and clones 231-HP1 and 231-HP2 both exhibiting similar high levels of psoriasin expression). Wild-type MDA-MB-231 and clone 231-neo (generated by transfection with the empty vector) do not express psoriasin. Jab1 and p27^{Kip1} antibodies were obtained from Santa Cruz Biotechnology, Inc. Hypoxic stimulation of cells was performed in a Forma Scientific Model 1025 Anaerobic System containing an atmosphere of 0.7% O_2, 5% CO_2, and 5% H_2 at 37°C for 24 h as we have described previously (27).

Immunoprecipitation and Western Blot. Human breast cancer cell lines expressing psoriasin (231-HP2 and MDA-MB-468) were lysed on ice in 25 mM HEPES (pH 7.7), 0.4 M NaCl, 1.5 mM MgCl$_2$, 2 mM EDTA, 1% Triton X-100, 0.5 mM DTT, and protease inhibitor mixture (Roche). Complexes were immunoprecipitated by Jab1 antibody/protein G-Sepharose (Pierce) at 4°C for 2 h. Binding and washes were performed in the same buffer, except the NaCl concentration was diluted 4-fold (28). Coimmunoprecipitated psoriasin protein was detected by immunoblotting using the psoriasin-specific antibody. Total protein lysates were extracted from the cell-line pellets in SDS-Isolation Buffer [50 mM Tris (pH 6.8), 20 mM EDTA, 5% SDS, 5 mM β-glycerophosphate, and a mixture of protease inhibitors (Roche)]. Protein concentrations were determined using the Micro-BCA protein assay kit (Pierce). Protein lysates were run on a 16.5% SDS-PAGE mini gel using Tricine SDS-PAGE to separate the proteins, and then transferred to 0.2 μm nitrocellulose (Bio-Rad). After blocking in 10% skimmed milk powder in Tris-buffered saline-0.05% Tween, blots were incubated with primary antibodies (~15 μg/ml in Tris-buffered saline-0.05% Tween) followed by incubation with appropriate secondary antibodies and visualization by incubation with Supersignal (Pierce) as per the manufacturer's instructions and exposure on X-ray films.

Reporter Gene Assay and Transcription of AP-1-dependent Genes. MDA-MB-231 parental cells and clones stably transfected with psoriasin were transfected with an AP-1-driven luciferase reporter gene (Stratagene) and a β-galactosidase expression vector, in triplicate experiments using Effectene (Qiagen). Luciferase was measured in cell lysates (Promega) 18 h after transfection and standardized to β-galactosidase activity (Promega). Total RNA from the MDA-MB-231 clones was isolated using TRIzol (Sigma) and reverse transcribed in duplicate from triplicate samples as described (4). Specific primers for VEGF, MMP13, and GAPDH were used for PCR as follows: VEGF-UPPER (sense) CGC AGA CGT GT AAA TGT TCC and VEGF-LOWER (antisense) AAG AAA AAT AAA ATG GCG AAT CC; MMP13-UPPER (sense) ATG CGG GGT TCC TGA T and MMP13-LOWER (antisense) CGC AGC AAC AAG AAA CAA; and GAPDH-UPPER (sense) ACC CAC TCC TCC ACC TTT G and GAPDH-LOWER (antisense) CTC TTG TGC TCT TGC TTG TTG G. Reactions were stopped during the log-linear stage of PCR amplification and samples electrophoresed through an agarose gel that was poststained with ethidium bromide for band visualization. Images were captured using an LCD camera and MCID software (Imaging Research, St. Catharine's, Ontario, Canada).

Immunohistochemistry. Cultured cells were grown on microscope slides for 24 h, and then fixed and processed as described previously (4). Immunohistochemical staining for psoriasin was performed essentially as described previously, using an automated tissue immunostainer (Ventana Medical Systems, Phoenix, AZ), and 3,3'-diaminobenzidine immunohistochemistry kit and bulk reagents supplied by manufacturer. Briefly, the staining protocol was set to "Extended Cell Conditioning" procedure, followed by 12 h incubation with primary antibody (concentration 1:3000) and 32-min incubation with secondary antibody. Positive staining was assessed by light microscopy.

Adhesion, Growth, and Invasion Assays. MDA-MB-231 clones were trypsinized from flasks that were 60–70% confluent. Cells (10,000) were plated in triplicate on three different days in 96-well plates having fibronectin, collagen I, or uncoated plastic surfaces (Becton Dickinson). After 1 h at 37°C, nonadherent cells were gently washed away with PBS. Adherent cells were stained with crystal violet, and their relative abundance determined by spectrophotometric absorbance. For growth assay, 1000 cells/well were plated in plastic 96-well plates in triplicate on three different days and allowed to grow for 18, 24, 48, and 72 h. Cells were stained with crystal violet and their relative abundance determined by spectrophotometric absorbance. Invasion assays were preformed in triplicate on a Matrigel-coated modified Boyden-invasion chamber (24-well plate inserts with 8-μm pores; Becton Dickinson). FBS DMEM (10%) was used as a chemoattractant in the lower chamber. Cells (350,000) were added to the upper chamber, and allowed 12 h to degrade the Matrigel and invade through the porous membrane. Cells that invaded and were adhering to the bottom of the membrane were stained with crystal violet. Invaded cells were visualized by light microscopy and numerated by counting the number of cells per high power field in five random fields.

In Vivo Mouse Studies. Breast cancer cells (four experimental groups comprising MDA-MB-231 parental cells, 231-neo control, 231-LP1, and 231-HP1) were grown in culture and then suspended in 0.2 ml of PBS at a concentration of 5×10^5 cells before injection into mammary fat pads of female nude mice according to a protocol approved by the University of Manitoba Animal Care Committee. Each experimental group included 5 animals, and two injections were sited bilaterally in each animal to achieve a total of 10 possible tumor sites per group. Tumor diameters were measured by calipers at weekly intervals, and the tumor volume was calculated from the formula: volume = 4/3 π (0.5 × smaller diameter^2 × 0.5 × larger diameter). The experiment was continued for up to 8 weeks at which time all of the animals were euthanized, and all of the injection sites, tumors, and multiple organ tissues (abdominal lymph nodes, lungs, liver, and spleen) were examined grossly for the presence of tumor. Representative tissue blocks from all of the primary injection sites and all of the organ sites suspicious for metastatic tumor were subsequently processed by 10% formalin fixation, paraffin embedding, and preparation of H&E-stained sections for light microscopic examination.

RESULTS

Identification and Confirmation of Psoriasin Interacting Proteins. We used full-length psoriasin fused to the GAL4 DNA-binding domain as bait in a yeast two-hybrid assay (20) and screened 1.74×10^7 clones from a normal human breast cDNA library. Among 4 true positive clones (26), 1 (Fig. 1a) contained almost the full protein sequence (amino acids 42–335) for Jab1. As shown in Fig. 1a, controls including unrelated bait (Rad18) and prey (Mad2) constructs, and empty bait and prey vectors did not show any activation of reporter genes. We noted that a Jab1-binding motif common to several Jab1 interacting proteins described recently is also contained within psoriasin (Fig. 1b), so we tested whether this region was necessary for the psoriasin-Jab1 interaction. As shown (Fig. 1a), only the COOH-terminal portion of psoriasin that contains this motif interacted with Jab1. To additionally confirm the psoriasin-Jab1 interaction in breast cancer cells, psoriasin was stably transfected into MDA-MB-231 cells, and coimmunoprecipitation experiments performed using Jab1 and psoriasin antibodies. Psoriasin-Jab1 protein complexes were detected in both psoriasin-transfected MDA-MB-231 cells (231-HP2) and the breast cell line MDA-MB-468 (which exhibits endogenous psoriasin expression) when Jab1 antibody was used for immunoprecipitation (Fig. 1c). However no psoriasin-containing complex was detected in control lanes in the absence of Jab1 antibody or protein G beads. Psoriasin-specific antibody immunoprecipitated psoriasin from cell lysates but was unable to coimmunoprecipitate Jab1 (data not shown), presumably because of the proximity and partial overlap of the epitope recognized by the antibody (amino acids 88–101 of psoriasin) and the proposed Jab1-binding domain (amino acids 57–89 of psoriasin).

Psoriasin and Jab1 Cellular Localization. We localized psoriasin and Jab1 in the MDA-MB-231 clones by immunohistochemistry. Jab1, like psoriasin, has been found previously to be both nuclear and cytoplasmic in cell types other than breast. In MDA-MB-231 cells and all 4 of the transfected cell lines (231-neo, 231-LP1, 231-HP1, and 231-HP2) Jab1 is expressed at comparable levels in the cytoplasmic compartment (Fig. 2, *right panel*). However, in all three of the psoriasin-expressing clones, 231-LP1, 231-HP1, and 231-HP2 (Fig. 2, *left panels*), there is a relative increase in Jab1 within the nucleus. However, the total amount of Jab1 protein as detected by Western blot is similar in all of the cell clones and does not change in the presence of psoriasin (Fig. 3a). Psoriasin can also be detected by immunoprecipitation of medium conditioned by 231-HP2 and MDA-MB-468
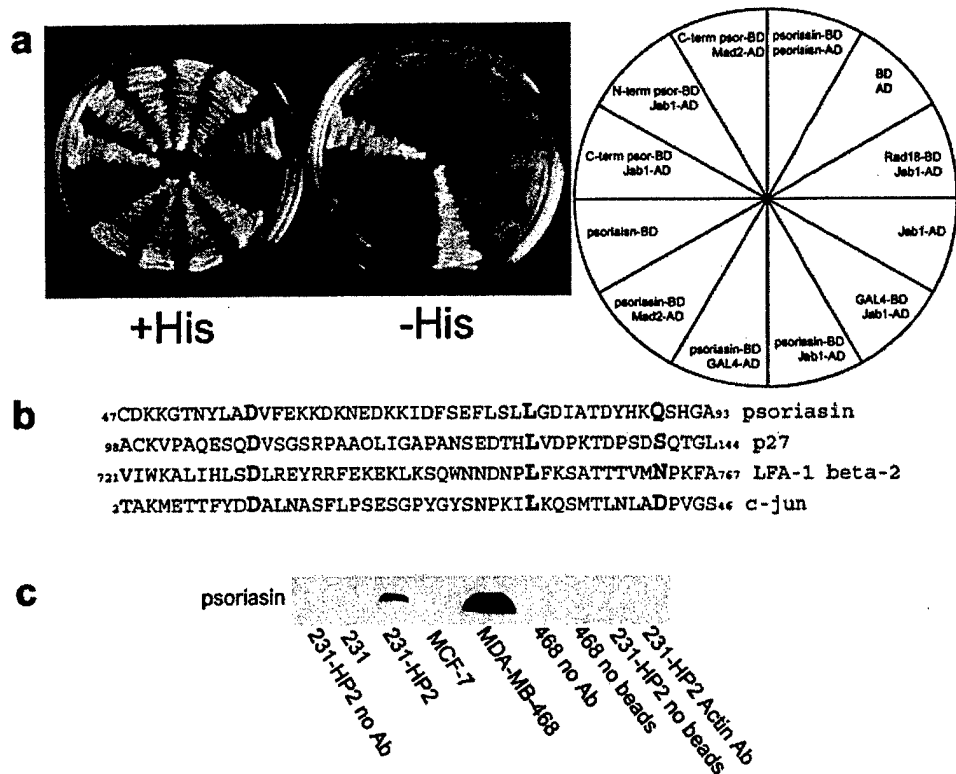
Fig. 1. Psoriasin specifically interacts with Jab1 in yeast two-hybrid assay and breast cancer cells. *a*, the psoriasin-Jab1 interaction was confirmed in yeast, by testing psoriasin fused to the GAL4-BD (binding domain) with selected proteins fused to the GAL4-AD (activating domain). Yeast plates (+ and − Histidine) are shown on the *left*, and the plating schema for each sector is shown on the *right*. Activation of reporter genes and colony growth is only present when the full-length psoriasin or the COOH-terminal half of psoriasin is combined with Jab1. *b*, the COOH-terminal half of psoriasin contains sequences similar to a Jab1-binding domain identified previously in p27^{Kip1}, LFA-1, and c-jun. *c*, psoriasin can be coimmunoprecipitated with Jab1 complexes from breast cancer cell lysates. Western blot using antipsoriasin antibody to detect psoriasin, which coimmunoprecipitated with Jab1, from a panel of breast cell lines (see "Materials and Methods").

b
47CDKKGTNYLADVFEKKDKNEDKKIDFSEFLSLLGDIATDYHKQSHGA93 psoriasin
94ACKVPAQESQDVSGSRPAAOLIGAPANSEDTHLVDPKTDPSDSQTGL144 p27
721VIWKALIHLSDLREYRRFEKEKLKSQWNNDNPLFKSATTTVMNPKFA767 LFA-1 beta-2
3TAKMETTFYDDALNASFLPSESGPYGYSNPKILKQSMTLNLADPVGS46 c-jun

c
psoriasin

cells (data not shown), suggesting that psoriasin is also secreted by breast cancer cells in culture.

Psoriasin Overexpression Influences Several Jab1-related Functions. Jab1 influences a number of cellular proteins. Among these, Jab1 effects the level of the negative cell cycle-regulating protein p27^{Kip1} by promoting the export of p27^{Kip1} from the nucleus to the cytoplasm and the subsequent degradation by the Ub-28S proteasome (29). Therefore, we first examined p27^{Kip1} expression in our MDA-MB-231 clones and found that psoriasin-overexpressing clones showed a consistent reduction in levels of p27^{Kip1} relative to wild-type and control cells (Fig. 3*a*).

To determine whether psoriasin influences other Jab1 functions in breast cancer cells we examined AP-1-dependent transcription in the MDA-MB-231 clones using an AP-1-driven luciferase reporter (Fig. 3*b*). AP-1 activity was increased in all 3 of the psoriasin-transfected clones in close proportion to the level of psoriasin expression (Fig. 3*a*). In the high psoriasin-expressing clones (231-HP1 and 231-HP2) there was a 6.5-fold increase in luciferase activity ($P < 0.0001$). These psoriasin-expressing cells showed no difference in total Jab1 levels assessed by Western blot, compared with non psoriasin-expressing controls (Fig. 3*a*). However, the effect on AP-1 activity is consistent with the redistribution and relative increase in nuclear Jab1 protein detected by immunohistochemistry (Fig. 2) and the findings of others (21). Expression of endogenous AP-1-dependent genes was next examined by RT-PCR (Fig. 3*c*). Psoriasin expression is also associated with an increase in mRNA levels of the endogenous AP-1-regulated genes VEGF (30) and MMP13 (31), and this increase is proportional to the levels of psoriasin in the MDA-MB-231 control and transfected cells.

Jab1 also interacts with HIF-1 (32) and enhances its activity. Expression of HIF-1 and the HIF-1-regulated gene *CAIX* (27) was examined by Western blot. Under hypoxic conditions (0.7% O$_2$), psoriasin-expressing clones showed a marked and higher induction of HIF-1 compared with control cells (Fig. 3*d, top panel*) and a parallel increase in CAIX protein (Fig. 3*d, middle panel*). However, it was

noted that CAIX expression was also increased in psoriasin-expressing 231-HP1 and 231-HP2 cells under normoxic conditions. The latter observation is consistent with the recent finding that CAIX can also be regulated by AP-1 (33) and indicates that a component of the CAIX induction seen under hypoxic conditions might be attributable to AP-1, given the involvement of AP-1 as well as HIF-1 in the cellular hypoxic response (34, 35).

Psoriasin Overexpression Influences Breast Tumor Progression *in Vitro*. We next looked for a relationship between psoriasin expression and biological end points relevant to tumor progression in breast cancer cells. The effect of psoriasin on growth of MDA-MB-231 cells was examined and found to be associated with a modest but significant increase in growth rate (Fig. 4*a*) of up to 1.3 fold ($P = 0.0009$). The influence of psoriasin on cellular adhesion, an important parameter of invasion, was measured in an *in vitro* assay. We observed a consistent reduction in cell-substrate adhesion (Fig. 4*b*) in psoriasin-expressing clones plated on plastic (0.42-fold reduction; $P < 0.0001$), collagen I (0.20-fold reduction; $P < 0.0001$), and fibronectin (0.18-fold reduction; $P < 0.0001$). The influence of psoriasin on invasion was then assessed in a modified Boyden chamber assay. There was a 1.4-fold increase in invasiveness in the high psoriasin-expressing clones ($P < 0.0001$) after 12 h (Fig. 4*c*), at which time there was no significant difference in growth (data not shown).

Psoriasin Overexpression Influences Breast Tumor Progression *in Vivo*. To determine whether psoriasin expression can also influence invasion and metastasis *in vivo*, psoriasin-overexpressing cells (231-LP1 and 231-HP1) and control cells (parental 231 and 231-neo) were injected into the mammary fat pad of nude mice, and the generation of tumors and metastasis was assessed (Fig. 5). Control cell lines (231 and 231-neo) generated tumors in 2 of 10 and 3 of 10 sites, respectively, after 8 weeks. These tumors were first noted between 2 and 3 weeks after injection, and increased slowly in size (Fig. 5*a*). Both psoriasin-expressing cell lines (LP1 and HP1) generated grossly detectable tumors in 7 of 10 and 6 of 10 sites. These tumors were also first noted between 2 and 4 weeks after injection but

Psoriasin Jab1

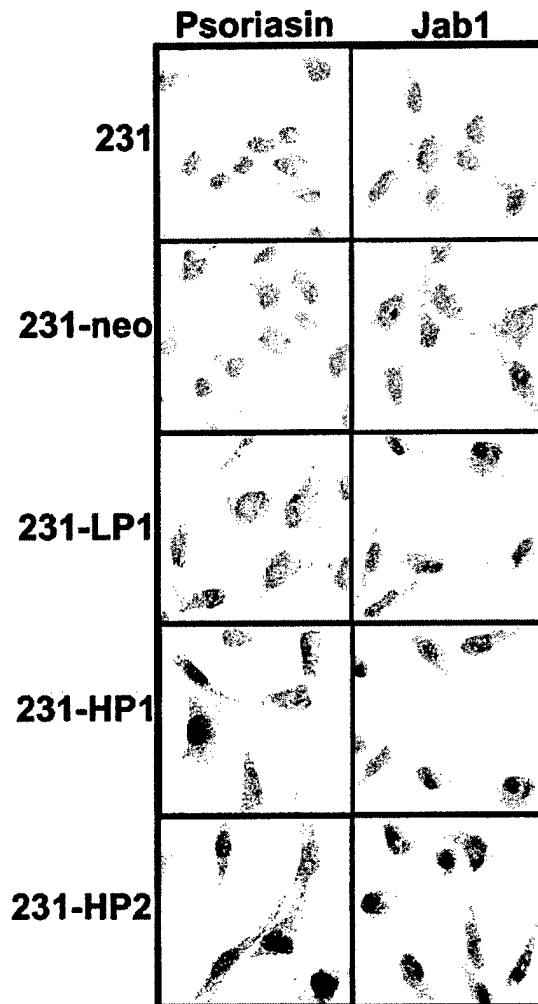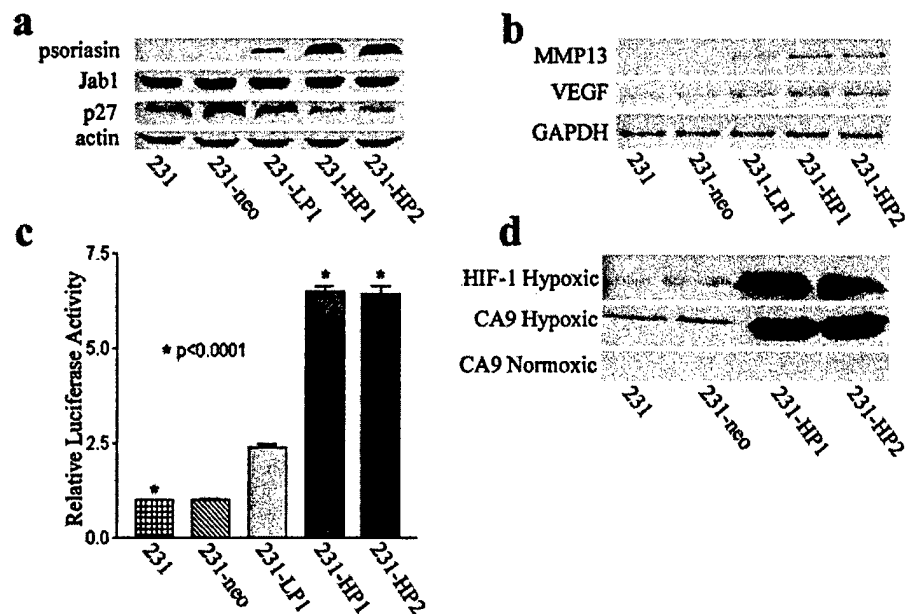
231

231-neo

231-LP1

231-HP1

231-HP2

Fig. 2. Psoriasin expression is associated with redistribution of Jab1 to the nucleus. Psoriasin and Jab1 were detected in cells by immunohistochemistry, and representative fields of each cell line are shown. *Left panels* show lack of psoriasin expression in control MDA-MB-231 cells and 231-neo cells, and the expression in the psoriasin transfected clones (231-LP1, 231-HP1, and 231-HP2). *Right panels* show comparable cytoplasmic Jab1 expression in each of the corresponding cell lines, but with enhanced nuclear Jab1 in all three psoriasin overexpressing clones.

increased rapidly in size (Fig. 5, *b* and *c*). By week 8 there was no difference in incidence or mean tumor size between parental 231 cells and 231-neo controls, or between the two psoriasin-expressing clones (Fig. 5*e*). However, both psoriasin-expressing clones were significantly different from both parental and neo-transfected control cells ($P = 0.017$ and $P = 0.024$, Mann Whitney; Fig. 5*f*). Overall mean[SD] tumor sizes (mm^3) for each experimental group were; MDA-231 = 21[11], 231-neo = 54[8], LP1 = 336[223], and HP1 = 370[270]. When control groups and psoriasin transfectant groups were combined, the mean[SD] tumor sizes (mm^3) were also significantly different: MDA-213 + 231-neo = 40[20] and LP1 + HP1 = 352[236] combined ($P = 0.0016$, Mann Whitney test). Microscopic examination of primary injection sites identified one additional microscopic tumor in the LP1 cell line group. The primary tumors derived from both control and psoriasin-expressing cells showed similar histological appearances. Expression of psoriasin was confirmed in representative tumors derived from psoriasin-transfected cell clones by immunohistochemistry (data not shown) and by Western blot (Fig. 5*d*). Psoriasin expression was only detected in tumors from psoriasin-transfected cells (although only a very weak signal was detected in the LP1 cell line). p27 expression was reduced in both psoriasin-transfected cell clone tumors. Grossly evident metastasis was identified and confirmed by microscopy in abdominal lymph nodes distant from the primary injection sites in 2 of 10 mice injected with psoriasin-overexpressing cells (both in the HP1 cell line group) compared with 0 of 10 mice in the control experimental groups.
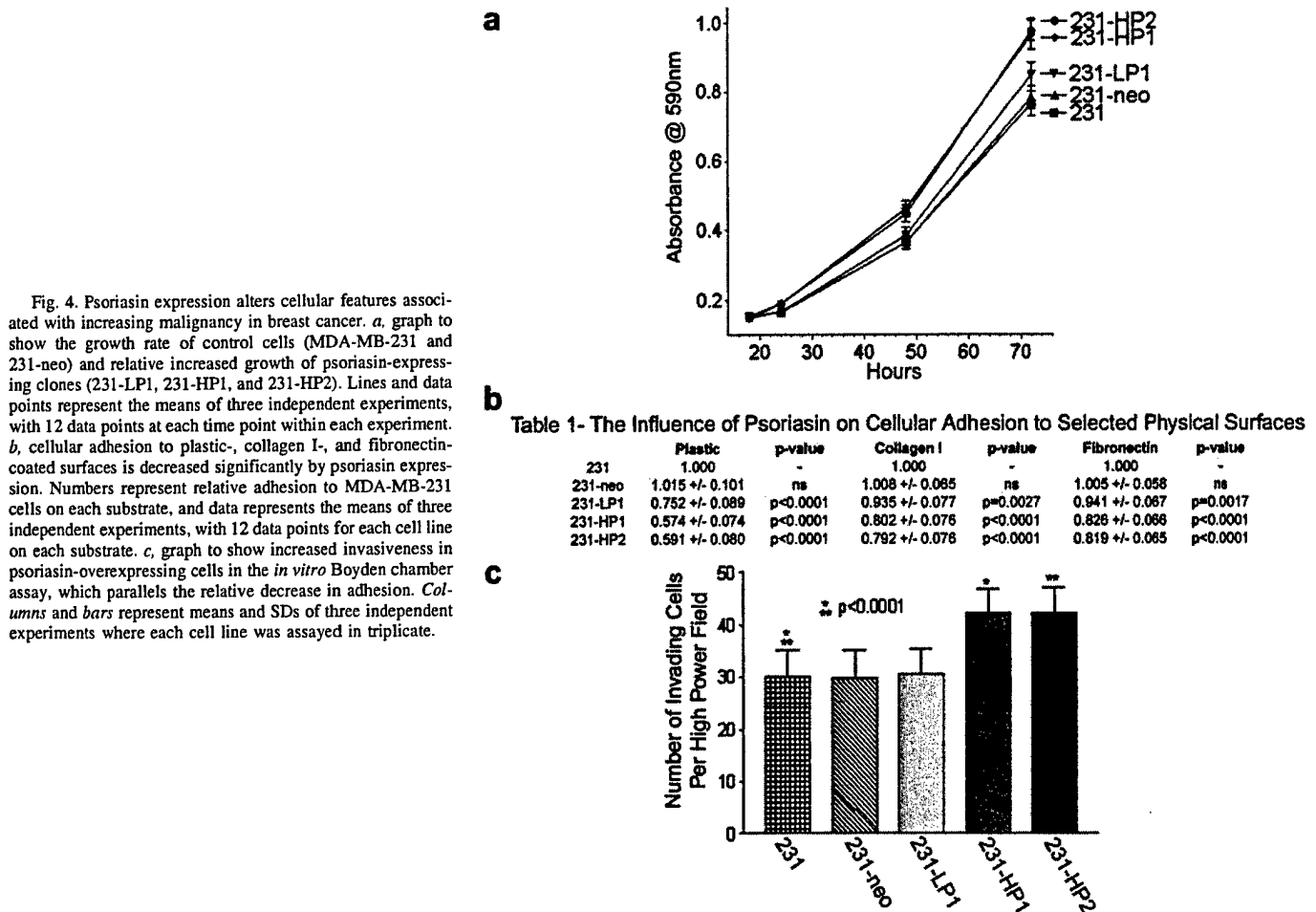
DISCUSSION

The transition from normal epithelium through DCIS to invasive breast cancer is likely to involve many complex processes that are influenced by dynamic changes in gene expression (36). Perhaps the most critical of these processes is the acquisition of the invasive phenotype (37) that occurs with the transition from DCIS to invasive disease, because this event transforms an otherwise local disease into one that is capable of distant spread to threaten the host. It is likely that some of those genes that show alterations in expression between preinvasive and invasive components of breast tissues may be relevant to the process of invasion and offer markers of risk of early tumor progression (36). In this study we demonstrate that the psoriasin gene,

Fig. 3. The biological effects of psoriasin are mediated through Jab1. *a*, Western blot showing absent psoriasin expression in MDA-MB-231 and control 231-neo cells, and the relative levels of psoriasin expression in 3 transfected clones (231-LP1, 231-HP1, and 231-HP2). The total amount of cellular p27^{Kip1} is reduced in psoriasin-expressing clones, whereas the total amount of Jab1 does not change. Actin is shown as a loading control. *b*, psoriasin is associated with an increase in AP-1 activity, as tested by transfection of an AP-1-driven luciferase reporter plasmid. The relative increase in luciferase activity is proportional to the level of psoriasin in the MDA-MB-231 control and transfected cells. *c*, RT-PCR assay to show that psoriasin is also associated with an increase in mRNA levels of the endogenous AP-1-regulated genes VEGF and MMP13 that is also proportional to the levels of psoriasin in the MDA-MB-231 control and transfected cells. *d*, Western blot to shown that psoriasin expression is associated with up-regulation of HIF-1 and induction of the HIF-1-regulated gene, CAIX. Under normoxic conditions there is an increase in CAIX expression in psoriasin-expressing clones. Under hypoxic conditions there is a marked increase in induction of both HIF-1 and CAIX in the psoriasin-expressing clones; *bars*, ±SD.

a
psoriasin
Jab1
p27
actin

b
MMP13
VEGF
GAPDH

c
Relative Luciferase Activity
7.5
5.0
2.5
0.0
* p<0.0001

d
HIF-1 Hypoxic
CA9 Hypoxic
CA9 Normoxic

a

Absorbance @ 590nm / Hours

231-HP2
231-HP1
231-LP1
231-neo
231

Fig. 4. Psoriasin expression alters cellular features associated with increasing malignancy in breast cancer. *a,* graph to show the growth rate of control cells (MDA-MB-231 and 231-neo) and relative increased growth of psoriasin-expressing clones (231-LP1, 231-HP1, and 231-HP2). Lines and data points represent the means of three independent experiments, with 12 data points at each time point within each experiment. *b,* cellular adhesion to plastic-, collagen I-, and fibronectin-coated surfaces is decreased significantly by psoriasin expression. Numbers represent relative adhesion to MDA-MB-231 cells on each substrate, and data represents the means of three independent experiments, with 12 data points for each cell line on each substrate. *c,* graph to show increased invasiveness in psoriasin-overexpressing cells in the *in vitro* Boyden chamber assay, which parallels the relative decrease in adhesion. *Columns* and *bars* represent means and SDs of three independent experiments where each cell line was assayed in triplicate.

b

Table 1- The Influence of Psoriasin on Cellular Adhesion to Selected Physical Surfaces

	Plastic	p-value	Collagen I	p-value	Fibronectin	p-value
231	1.000	-	1.000	-	1.000	-
231-neo	1.015 +/- 0.101	ns	1.008 +/- 0.065	ns	1.005 +/- 0.058	ns
231-LP1	0.752 +/- 0.089	p<0.0001	0.935 +/- 0.077	p=0.0027	0.941 +/- 0.067	p=0.0017
231-HP1	0.574 +/- 0.074	p<0.0001	0.802 +/- 0.076	p<0.0001	0.826 +/- 0.066	p<0.0001
231-HP2	0.591 +/- 0.080	p<0.0001	0.792 +/- 0.076	p<0.0001	0.819 +/- 0.065	p<0.0001

c

Number of Invading Cells Per High Power Field

$p < 0.0001$

231 / 231-neo / 231-LP1 / 231-HP1 / 231-HP2

which is highly expressed in DCIS and associated with poor prognostic factors when expressed in invasive disease, can enhance growth, adhesion, and invasiveness of a breast cell line in *in vitro* assays and tumorigenicity in nude mice *in vivo*. Furthermore, we describe a potential mechanism for these effects through a direct interaction between psoriasin and the multifunctional intracellular protein Jab1 (21).

Jab1 was originally identified in mammalian cells as a factor influencing c-jun transcription of AP-1-regulated genes (21). It soon became clear that Jab1 was also a component (CSN5) of a multimeric protein complex (22, 23). The CSN/COP9 signalosome had been studied previously in other systems and shown to be involved in protein degradation via the Ub-26S proteasome (24, 25). Jab1 has since been shown to be involved in a diversity of interactions with components of cell signaling pathways in *in vitro*, yeast, and human cell line model systems. These interactions appear to result in either translocation of Jab1 from cytoplasm to nucleus (integrin LFA-1 [38], erbB-2 [39] signaling), enhanced activity of transcription factors (including c-jun/AP-1 [21], HIF-1 [32], steroid receptors and cofactors [40, 41]) or the promotion of degradation of the interacting protein (including Smad4 [42], p53 [43], HIF-1 [32], MIF1 [28], and p27^{Kip1} [29, 43]), often but not always associated with translocation from nucleus to cytoplasm. However, the physiological relevance of some of these interactions, and specifically in the context of breast epithelial cells, is mostly unknown.

In ovarian tumors, increased nuclear Jab1 is associated with progression and poor outcome (44), and altered Jab1 has also been implicated in renal cancer (45). A direct role for Jab1 in breast cancer

has not been identified previously; however, several proteins including p53 and erbB-2, which are known to interact with or to influence Jab1, are altered at an early stage within high-risk DCIS (46–49) and may exert some of their effects through Jab1. The interaction between psoriasin and Jab1 also has the potential to directly facilitate several aspects of early tumor progression. We have shown here that overexpression of psoriasin is associated with translocation of Jab1 to the nucleus, alterations in expression of several Jab1 "downstream" genes, and increased proliferation, altered response to hypoxia, and promotion of invasion. Increased proliferation may be specifically attributable to increased AP-1 activity and down-regulation of the cell cycle inhibitor p27^{Kip1} in this model. Alteration of Jab1 might also lead to increased activation of estrogen receptor and progesterone receptor, and up-regulation of cyclin D1 and alteration of transforming growth factor β signaling in other cell models (39, 50, 51), but these aspects of Jab1 function remain to be examined in the context of breast cancer. Increased capacity to survive hypoxic stress may occur through augmented HIF-1 activity and hypoxic response. Increased invasiveness may result from activation of AP-1 and HIF-1-dependent genes (52, 53), such as matrix metalloproteinases and VEGF, which are already implicated as critical factors in breast tumor progression (37, 54).

The estrogen receptor-negative MDA-MB-231 breast cell line was selected to reflect the context of psoriasin expression that we and others have observed previously in breast tumors *in vivo* (2, 4). The modest although significant increase in proliferation and invasiveness seen in our *in vitro* assays may reflect the fact that this cell line is already a highly proliferative and invasive cell in *in vitro* assay. More
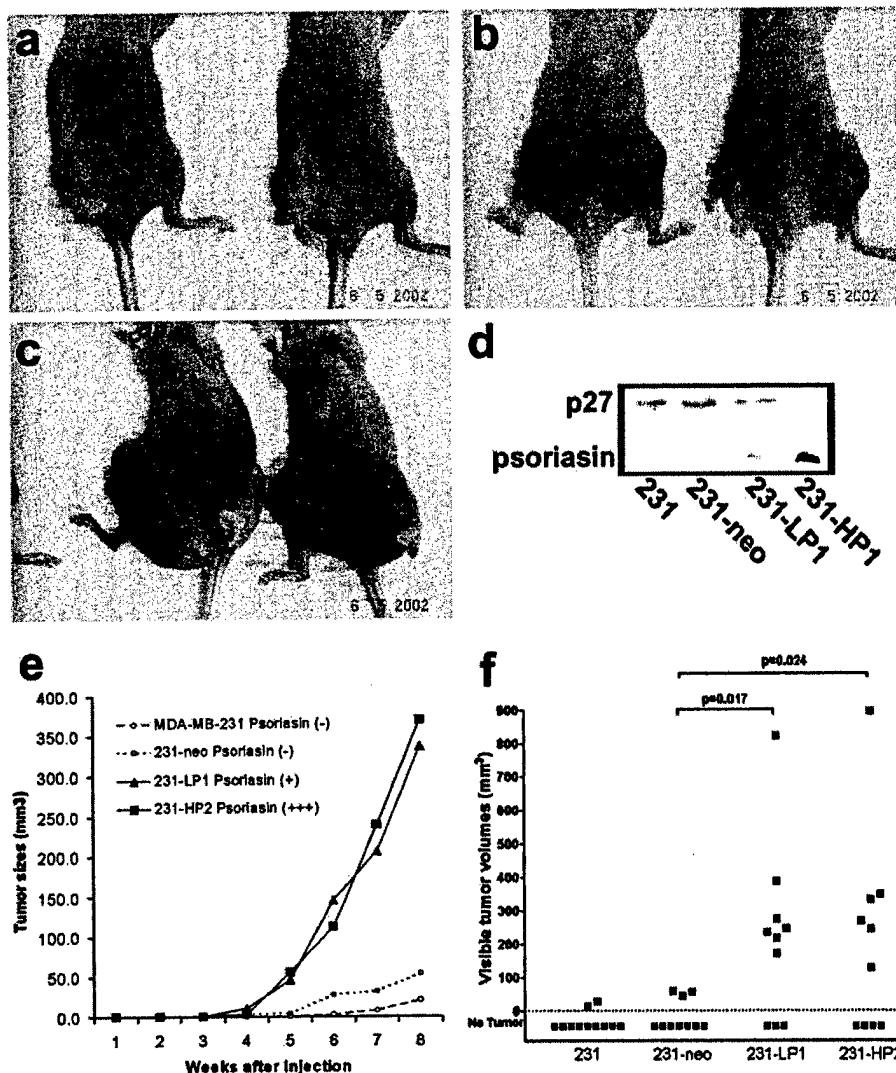
Fig. 5. Effect of psoriasin expression on tumor growth in nude mice. Groups of 5 mice for each cell line received an injection of 5×10^5 cells into the mammary fat pads. The *top panels* (*a–c*) show representative mice at 8 weeks from each treatment group that received (*a*) MDA-MB-231 cells, (*b*) 231-LP1 cells, or (*c*) 231-HP2 cells. *d*, expression of psoriasin and p27 protein determined by Western blot on extracts from representative tumors. *e*, relative growth curves for mice in each group. *Lines* and *data points* indicate the mean tumor volumes at each time point. *f*, distribution of tumor volumes at 8 weeks. Statistical significance was determined by Mann-Whitney test.

striking increases in growth and invasiveness were observed *in vivo* in the nude mice experiments, where metastasis was also associated with psoriasin-expressing tumors. This difference is consistent with the anticipated effects of enhanced metalloproteinase and VEGF expression on extracellular matrix and angiogenesis, spheres of influence that are not adequately replicated in *in vitro* assays, and has been observed by others studying the effects of overexpression of VEGF in breast cell lines (55). Nevertheless, additional detailed studies will be necessary to confirm the direct relationship and functional role of these specific factors in the enhanced growth and invasiveness seen in this model *in vivo*.

Alteration of Jab1 activity in tumors could be attributable in part to alterations in either the cytoplasmic-nuclear distribution (Refs. 38, 45, 56; as appears to be the case for the effect of psoriasin), the ratio of free Jab1:COP9-associated Jab1 (56), competition between different interacting proteins (42), or direct elevation of Jab1 expression and activation. The relevance of these potential mechanisms of action to breast cancer remains to be resolved, both for psoriasin and several other Jab1-interacting proteins. Nevertheless, it has been demonstrated that the many important activities of Jab1 can be influenced by competition between different interacting proteins (42). For example, p53 can compete with and down-regulate Jab1 activation of c-jun (57), and inhibition of Jab1 causes reciprocal up-regulation of p53 (42) and down-regulation of c-jun in HeLa cells (57). It is also

interesting to note that the chemokine MIF can exert the opposite effect on Jab1 to psoriasin (28) with respect to modulation of AP-1 activity and $p27^{Kip1}$ expression. This raises the question of whether these different chemokine molecules might compete to modulate Jab1 activity.

Whereas our data support the involvement of Jab1 in mediating many of the biological actions of psoriasin, additional experiments will be needed to confirm that a direct interaction occurs between the putative Jab1 binding motif (29) on psoriasin and the Jab1 protein, and that direct alterations of Jab1 indeed exert effects on these specific target genes and pathways. It is also possible that some of the functions of psoriasin are mediated through other pathways (58). For example, it has been shown that other secreted S100 proteins (S100B and S100A12) can bind to and stimulate the receptor for advanced glycation end products, leading to activation of intracellular signaling pathways including up-regulation of ras, mitogen-activating protein kinase and nuclear factor $\kappa\beta$ in immune cells (59, 60). Expression of receptor for advanced glycation end products is also associated with invasion in gastric carcinoma (61) and is functionally involved in metastasis (62). Unlike some other S100s with chemokine activities such as S100A9 and S100A12, which are expressed by both epithelial and stromal inflammatory cells (63), expression of S100A7 (psoriasin) is restricted to epithelium, at least in skin and breast. However,

psoriasin is also secreted and could potentially interact with cell surface receptors on immune or epithelial cells.

In summary, we have shown that psoriasin can contribute to breast tumor progression and that its action may be mediated, at least in part, through Jab1. Although other important cellular proteins also influence and may compete for Jab1, psoriasin is one of the most abundant proteins in high-risk DCIS (2) and is likely to exert an important effect on Jab1 activity in breast tumor cells at an early stage of tumor progression. Thus, therapies aimed at modulating the effect of psoriasin may have important potential in the treatment of early breast cancer.

ACKNOWLEDGMENTS

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S100A7 (Psoriasin) expression is associated with aggressive features and alteration of Jab1 in Ductal Carcinoma In-Situ (DCIS) of the breast

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3. The abbreviations used are: DCIS, ductal carcinoma in-situ; IHC, immunohistochemistry; RT, room temperature; ns, not significant; ER, estrogen receptor.

Key words: breast cancer, Jab1, p27, progression.

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ABSTRACT

Puropse: S100A7 expression in invasive carcinoma is associated with a worse prognosis which may be mediated in part through an interaction with the multifunctional cell signaling protein Jab1. We have investigated the relationship between expression of S100A7 and progression from DCIS to invasive carcinoma.

Experimental Design: To investigate the relationship between expression of S100A7 and progression from DCIS to invasive carcinoma, we selected 139 cases of DCIS (46 cases with and 93 cases without associated invasive carcinoma) and assessed S100A7 expression by immunohistochemistry. In a subset of cases, the expression and localization of Jab1 and $p27^{kip1}$ was also determined.

Results: 67/139 (48%) of DCIS lesions expressed S100A7 and were associated with ER negative status (p=0.0003), higher nuclear grade (p<0.0001) and necrosis (p<0.0001). S100A7 expression was also associated with an increase in nuclear Jab1 (n=43, p=0.0019) and reduced $p27^{kip1}$ (n=47, p=0.0168). In those cases associated with invasive carcinoma, there was a significant reduction in S100A7 between in-situ and invasive components (n=46, p=0.0007, Wilcoxon test). In pure DCIS cases treated by local excision, there was no difference in frequency of S100A7 expression between patients with recurrence of DCIS (n=9) and those without recurrence (n=36).

Conclusions: Consistent with our previous findings in invasive disease, these results confirm that S100A7 expression in DCIS correlates with poor prognostic features. While S100A7 may not be a marker for recurrence of DCIS, it may influence progression to invasive carcinoma in a subset of cases and this may be mediated through its interaction with and influence on Jab1.

INTRODUCTION

We previously identified S100A7 (psoriasin) as a gene expressed within pre-invasive ductal carcinoma in-situ (DCIS) that can be downregulated in invasive carcinoma[1]. Later studies have confirmed this observation and revealed that S100A7 is amongst the most highly expressed genes in DCIS[2,3] relative to both normal tissue and invasive carcinoma. A similar pattern of expression is also seen in relation to skin tumorigenesis where S100A7 is highly expressed in preinvasive squamous cell carcinoma and often downregulated in the adjacent invasive component[4].

We have now identified a potential functional relationship between S100A7 and tumor progression and a mechanism of action through an interaction with the multifunctional intracellular signaling protein Jab1 (c-jun activation domain binding protein 1)[5,6]. Overexpression of S100A7 in breast cancer cells is associated with increased malignancy and with several changes in gene expression that are compatible with an alteration in Jab1 activity[7]. The latter include a relative increase in nuclear Jab1 and decrease in the levels of the cell-cycle inhibitor p27[kip1][7,8].

Expression of S100A7 is highest in DCIS but only small numbers of cases have been examined[1-3]. S100A7 is also expressed in some invasive breast tumors[2,9], where S100A7 is associated with poor prognostic factors and with poor outcome. Our aim was to determine the frequency of expression of S100A7 in a large series of DCIS, its relationship to Jab1 and p27[kip1] expression, and to progression and clinical outcome in DCIS.

MATERIALS AND METHODS

Tissue Specimens.

Cases of pure ductal carcinoma in-situ of the breast were selected by review of breast surgical resections that occurred from 1981-1999 at the Health Sciences Center and St. Boniface Hospitals, Winnipeg Manitoba. Cases of DCIS associated with invasive carcinoma were selected from the Manitoba Breast Tumor Bank[10] which operates with the approval from the Faculty of Medicine, University of Manitoba, Research Ethics Board. A total of 93 cases of pure DCIS and 46 cases of DCIS with invasive carcinoma were identified and classified into histological grades on the basis of the predominant grade present in the tissue section studied for gene expression according to the Van Nuys grading system[11]. The presence of intraductal necrosis was evaluated in hematoxylin and eosin stained sections by light microscopy. ER status was determined by immunohistochemistry analysis as described below.

The clinical-pathological features of the entire series are shown in Table 1. The predominant DCIS growth pattern or type was as follows; 53% solid, 27% cribriform, 14% comedo, 7% micropapillary. Amongst the DCIS- cases primary surgical treatment was local excision in 45 patients and was mastectomy in the other 47 patients. Amongst the former group 27 patients had surgery alone, 18 patients received adjuvant local radiation therapy and 11 received adjuvant hormone therapy. Clinical followup for the 45 patients treated by local excision was obtained by chart review and search of the Manitoba Cancer Registry. Recurrence in the same breast occurred in 9 cases (in 8 cases as DCIS and in 1 case as DCIS with microinvasive carcinoma) from 9 to 50 months after initial diagnosis. For those cases with no recurrence the median followup time was 66 months (range from 23-184 months).

Immunohistochemistry.

Five micron serial sections were cut from a representative formalin fixed paraffin embedded archival tissue block from each tumor. Immunohistochemical staining for S100A7, ER, Jab1 and p27[kip1] was performed as previously described[7,12], but using an automated tissue immunostainer (Ventana Medical Systems, Phoenix, AZ) and DAB immunohistochemistry kit (ABC method, Ventana). Briefly, the staining protocol was set to "Extended Cell Conditioning" procedure, followed by 12 hours incubation with primary antibody prior to incubation with secondary antibody and detection. Concentrations and sources of the primary antibodies were 1:1000 for S100A7, 1:50 for ER (6F11, Novocastra), 1:200 for Jab1 (FL-334, Santa Cruz) and 1:1000 for p27[kip1] (Becton-Dickenson) antibodies using a similar protocol.

Assessment of S100A7, ER, Jab1, and p27[kip1] staining.

Immunostaining for all genes was assessed by light microscopy performed by a single pathologist (P.H.W.) independently of the pathological assessment. S100A7, p27[kip1] and Jab1 expression (both nuclear and cytoplasmic staining assessed separately) and ER expression (nuclear staining) was scored as positive. The intensity (0=no staining, 1=weak staining; 2=moderate staining; and 3=strong staining) and the percentage of neoplastic epithelial cells showing expression within the tissue section (0-100%) was scored. The product of the intensity and the percentage gave a final semi-quantitative immunostaining score (IHC scores ranging from 0-300). For categorical statistical analysis cases with S100A7 and ER IHC scores >0 were regarded as positive. Statistical analysis was performed with Prism Graphpad software and using spearman correlation, t-test, Mann Whitney and Wilcoxon rank sum tests as appropriate.

RESULTS

Evaluation of S100A7 and pathological features within DCIS.

In the entire series of 139 DCIS breast cases studied by immunohistochemistry (IHC) 67/139 (48%) of DCIS lesions expressed S100A7. Expression was predominantly cytoplasmic and occasionally nuclear, was restricted to epithelial tumor cells and was not observed in adjacent normal ducts or lobules as we have previously described[9]. S100A7 positive cases included 43/93 (46%) of pure DCIS cases and 24/46 (52%) of DCIS cases associated with invasive carcinoma. In 16 cases (11.5%) expression of S100A7 was present in 50% or more of the tumor cells. However in several cases expression was only focal and was limited to one or two cells within the DCIS lesion. The S100A7 IHC scores ranged from 0 to 270 (mean 28, standard deviation 62, median 0). Expression was not seen in adjacent normal tissues. Representative examples of S100A7 staining are illustrated in Figure 1.

S100A7 expression determined by S100A7 IHC score did not correlate with DCIS growth pattern or type, but correlated with high nuclear grade (r=0.54, p<0.0001, Spearman test), ER levels (r=-0.36, p<0.0001), and extent of necrosis. (r=0.44, p<0.0001). Similarly, when prognostic factors were considered as categorical variables S100A7 expression was also significantly associated with high grade (p<0.001 Mann Whitney test), and ER negative status (p<0.001) and the presence of necrosis (p<0.0001, Table 2).

Relationship between S100A7, Jab1, and p27^{kip1}

Expression of Jab1 and p27^{kip1} was also assessed in DCIS in a subgroup of 47 cases where there was sufficient material available. These included 26 cases of pure DCIS and 21 cases of DCIS associated with invasive carcinoma. S100A7 expression levels correlated positively with nuclear Jab1 (r=0.44, p=0.029, Spearman test) and inversely with cytoplasmic p27^{Kip1} (r= -0.32,

p=0.028). When nuclear, cytoplasmic, and total combined expression of Jab1 was considered relative to S100A7 status, S100A7 positive DCIS was associated with higher relative expression of nuclear Jab1 (p=0.0019), but no difference in cytoplasmic or overall Jab1 expression. Conversely, S100A7 positive DCIS was associated with significantly lower overall expression of p27^{kip1} (p=0.0168, t-test), and similar but not significant trends towards lower nuclear and cytoplasmic expression (Figures 1&2).

Relationship between S100A7 and progression to invasive carcinoma.

In the DCIS series, 46 cases were associated with concurrent invasive carcinoma, allowing assessment of S100A7 expression in matched DCIS and invasive components. Amongst these S100A7 was expressed in the DCIS component in 24 cases but was often expressed at significantly lower levels in the matching invasive components (p<0.0001, Wilcoxon test). S100A7 expression in the invasive component was either absent (9 cases) or expressed at lower levels (12 cases) or the same level (3 cases) as the DCIS (Figure 3). In one case (1/46), S100A7 was expressed only in the invasive component. In those cases that showed the highest levels of S100A7 expression in the DCIS (>=50% of the DCIS component positive), 10/11 cases showed a 50% or greater reduction in IHC score in the invasive component. There was no significant change in Jab1 expression or localization between DCIS and invasive components.

S100A7 and recurrence in DCIS cases treated by local excision.

Amongst the 93 pure DCIS cases, 45 patients had been treated by local excision. In this subset, 36 (80%) did not develop a recurrence and 9 (20%) developed a recurrence. In cases that recurred, the recurrence was DCIS in 8/9 cases and DCIS with an invasive component in 1/9 cases. There was no significant difference in patient age, excision margin status, or frequency of additional radiation or hormonal treatment between the subgroups with or without recurrence.

Similarly there was no significant difference between the clinical-pathological characteristics of these DCIS cases associated with different outcome, although lesion size could not be accurately determined and was not included in the analysis. The subset with recurrence showed more frequent necrosis (66% vs. 51%) and ER negative status (44% vs. 20%), and a lower proportion of cases that were treated by radiotherapy (13% vs. 27%), however none of these differences was statistically significant. There was also no significant difference in the frequency of S100A7 expression between those cases with and without recurrence.

In 7 DCIS cases with recurrence the tissue blocks from the recurrence was also available to be assessed for S100A7 expression. While both nuclear grade and the presence of necrosis were concordant in only 4/7 (57%) cases, ER status was concordant in 5/6 (83%) and S100A7 status was concordant in 7/7 (100%) cases.

DISCUSSION

The S100 gene family encodes small proteins that share EF-hand helix-loop-helix domains that are important for their function as calcium binding proteins[13]. Several S100 genes are altered in neoplasia, and specifically in breast cancer[14], including S100A4, which can influence the metastatic phenotype in mammary cell lines and is associated with poor outcome in invasive breast tumors[15-17].

S100A7 (psoriasin) was first identified as a highly abundant cytoplasmic and secreted protein induced in abnormally differentiating squamous epithelial cells derived from epidermis of skin involved by psoriasis[18] The association with psoriasis has suggested a role for S100A7 either in keratinocyte differentiation or as a chemotactic factor[19-21].

A possible role for S100A7 in breast cancer first emerged when it was identified as a cDNA downregulated in breast nodal metastasis[22]. We also identified S100A7 as a differentially

expressed gene between the earlier stage of DCIS and invasive carcinoma[1]. Although previous studies have been limited to small numbers of DCIS lesions, S100A7 has emerged from subtraction hybridisation[1], SAGE[2,23] and proteomic analyses[3] as amongst the most highly expressed genes in some high grade DCIS. By comparison S100A7 expression is relatively low in normal, benign and atypical proliferative ductal lesions, and in invasive carcinoma[1]. When S100A7 is expressed in invasive tumors it is associated with markers of poor prognosis, including a strong association with estrogen receptor negative status[9]. Amongst this estrogen receptor negative subset of invasive carcinomas, S100A7 is an independent prognostic factor associated with poor outcome[24].

Taken together, these observations support the hypothesis that S100A7 plays a functional role in breast tumor progression and the development of the invasive phenotype. S100A7 therefore has potential to provide a prognostic marker in DCIS. In support of this hypothesis we have recently shown that S100A7 can promote several features of malignancy in an already invasive ER negative breast cell line, both in-vitro and in-vivo[7]. Furthermore, we have identified a potential mechanism for these effects through an interaction between S100A7 and the multifunctional 'c-jun activation domain binding protein 1'. Jab1 was originally identified in mammalian cells as a factor influencing c-jun transcription of AP-1 regulated genes[5,25]. It is now know that Jab1 is a component of both a multimeric protein complex (CSN5 within the CSN/COP9 signalosome) and also the lid unit of the Ub-26S proteasome[6,26].

Studies in cell lines suggest that Jab1 is involved in a diversity of interactions with components of cell signalling pathways[25,27-33]. Jab1 therefore has the potential to directly facilitate several aspects of early tumor progression through alteration of multiple cellular properties including promoting degradation of p27^{kip1} [32]. In studies of human tumors, increased nuclear Jab1 has been associated with poor outcome in invasive carcinomas of both breast and ovary, and in lymphoma[34-36]. This is consistent with the observation that many of the interactions between Jab1

and signalling molecules and pathways appear to result in translocation of Jab1 from the cytoplasm to the nucleus in cell lines[7]. We have previously observed that expression of S100A7 induces a relative increase in nuclear Jab1 in a breast cell line, without affecting the overall level of expression, and a concurrent reduction in p27^{kip1} protein[7]. In the present study we have shown that S100A7 expression in DCIS is also associated with higher nuclear expression of Jab1, without a significant increase in overall expression, and reduced p27^{kip1}. These findings are consistent with a recent study that also found high nuclear Jab1 expression is inversely correlated with expression of p27^{kip1} in invasive breast carcinoma[35]. The frequency of nuclear Jab1 expression in DCIS appears to be similar to that reported in invasive carcinomas, with over 85% of DCIS exhibiting Jab1 expression in >30% of the tumor cells. However we detected some cytoplasmic Jab1 expression in most DCIS cases (>80%), and this was often comparable to the nuclear signal. Comparable levels of cytoplasmic Jab1 expression was noted in the invasive components of some cases, however future studies will be needed to determine if this difference is attributable to different Jab1 antibodies and immunostaining methods, or alterations in Jab1 expression inherent to the stage of tumor progression.

Progression of DCIS may result in recurrence in the form of DCIS or invasive disease[37]. In this series the majority of recurrence events were DCIS lesions and we found no relationship with S100A7 expression. Recurrence in the form of DCIS may be derived from residual disease attributable to incomplete surgery of the original focus of disease, from emergence of distant lesions in an originally multifocal disease, or de-novo disease arising in previously normal remaining tissue. Molecular studies suggest that the former may often occur[37,38]. Our finding that the S100A7 phenotype is reliably conserved between primary lesions and recurrences is also consistent with this view.

While the small numbers of recurrent tumors limits firm conclusions, our results suggest that S100A7 does not influence those factors that might influence the resection margin status, such as

the size or extent or subtype and growth pattern of DCIS. However the association of S100A7 with established poor prognostic factors in DCIS, and the occurrence of altered expression in associated invasive components are consistent with a role in progression to invasive disease. Our results also extend and confirm recent observations in invasive carcinoma, showing that increased S100A7 correlates with increased nuclear Jab1 and reduced p27^{kip1}, and further support the potential importance of S100A7 as a factor in mediating progression from DCIS.

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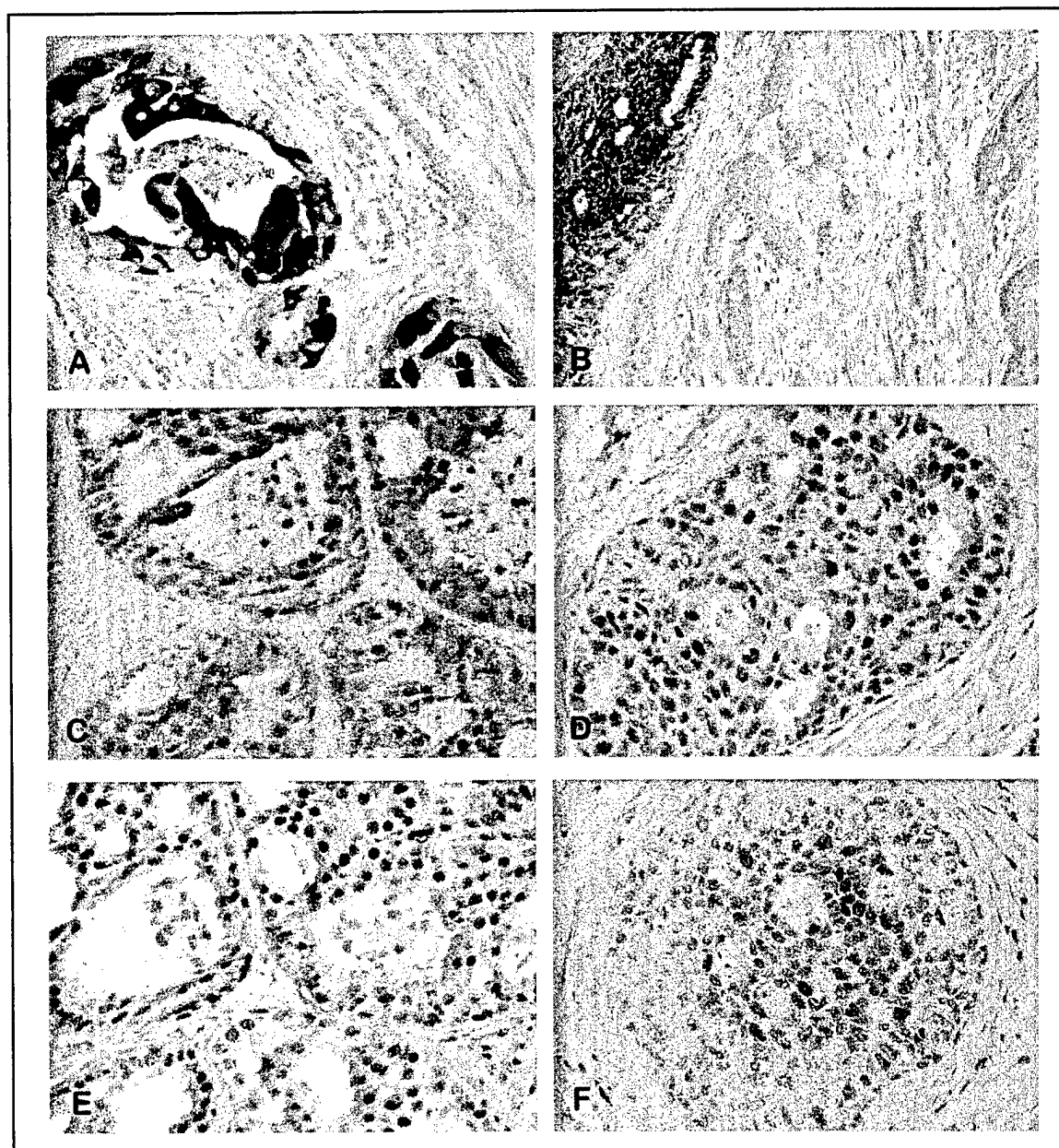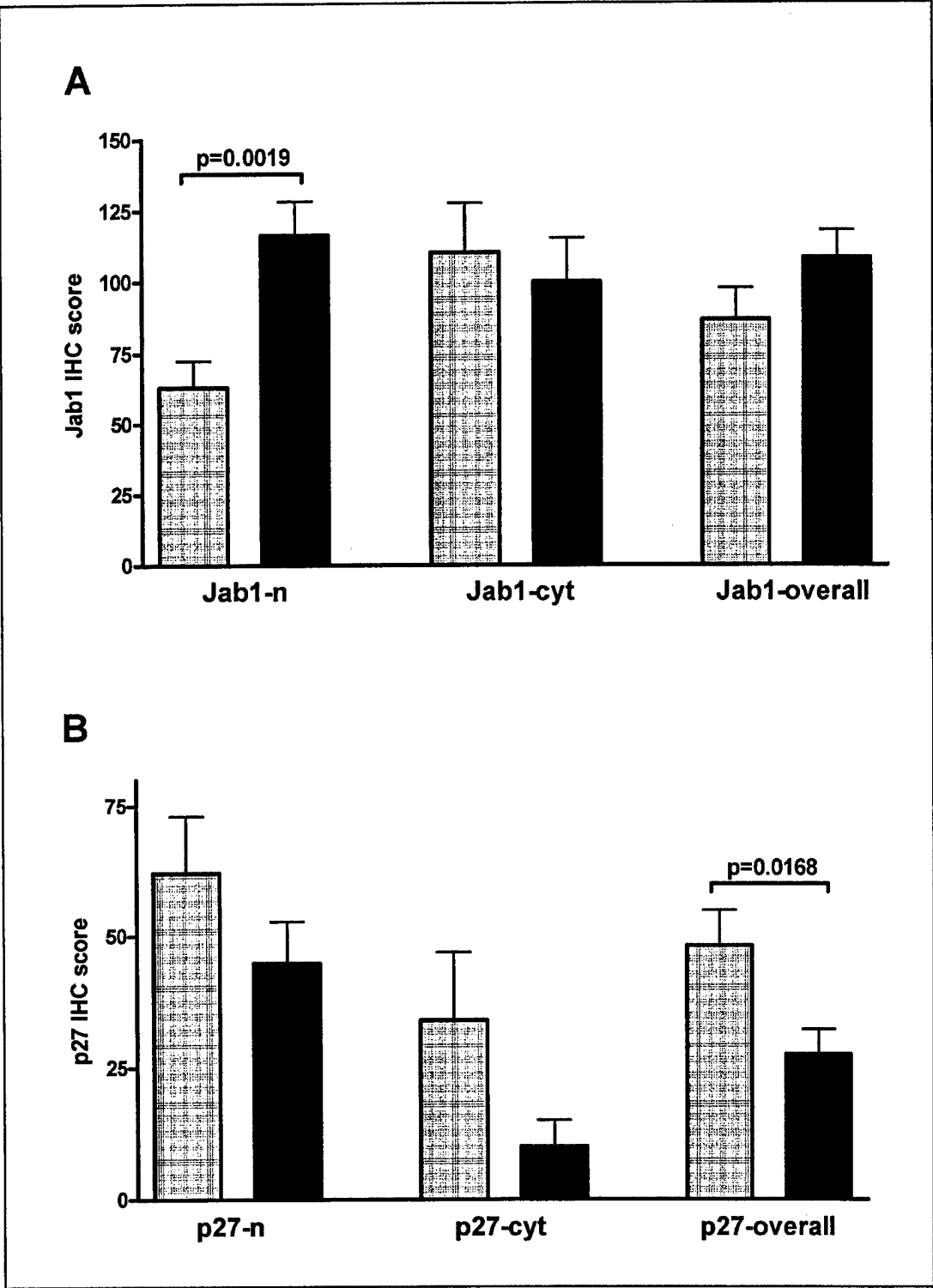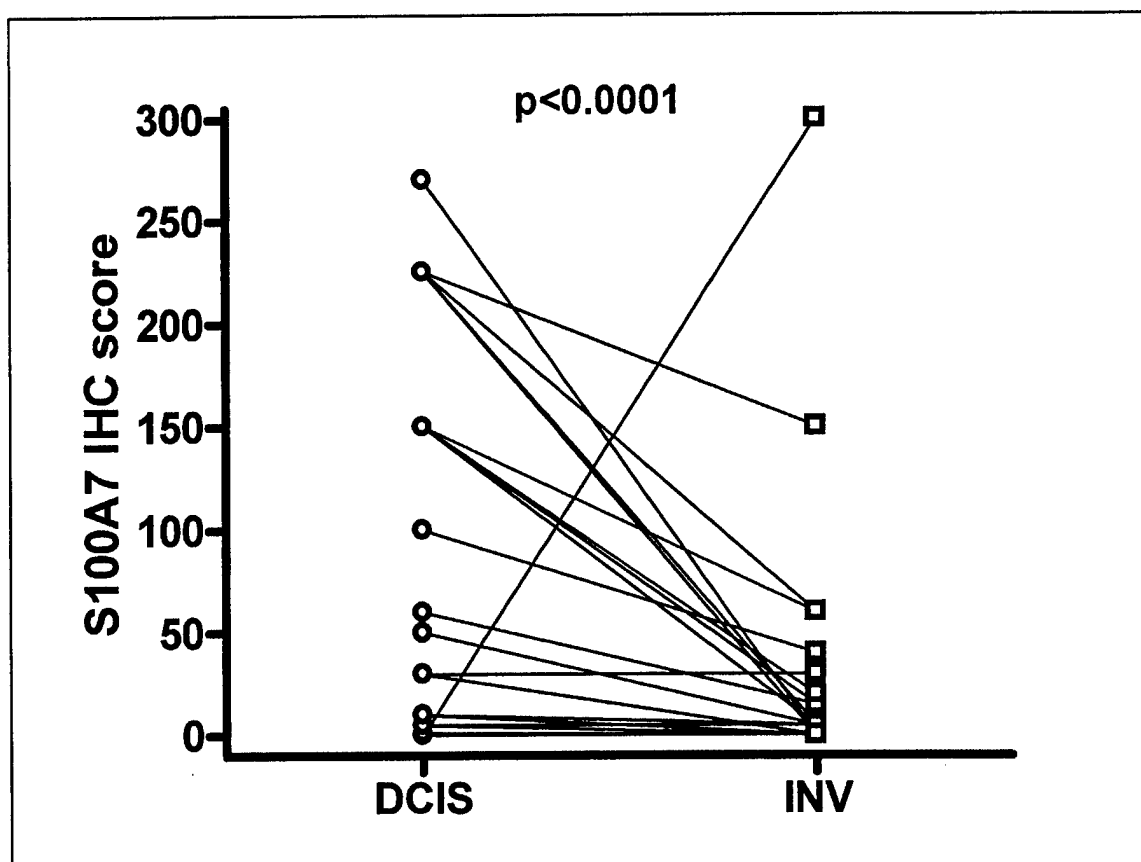
Figure 1.

Figure 2

Figure 3.

LEGENDS TO FIGURES

Figure 1. S100A7, Jab1, and p27^{kip1} expression in DCIS detected by immunohistochemistry. Upper panels show high levels of S100A7 expression in pure DCIS (A) and in DCIS associated with invasive carcinoma (B). Note reduced expression in invasive component (B, right) relative to in-situ component (B, left). Middle panels show Jab1 expression in S100A7 negative (C) and positive (D) DCIS cases. Lower panels show high (E) and low (F) p27^{kip1} expression in the same two DCIS cases.

Figure 2. Jab1 and p27^{kip1} expression relative to S100A7 status. A) Upper graph illustrates higher Jab1 nuclear expression (Jab-n) in S100A7 positive (black columns) versus negative (grey columns) cases, but similar cytoplasmic (Jab-cyt) and overall expression. B) lower graph illustrates lower overall p27^{kip1} expression in S100A7 positive cases. Columns represent mean IHC scores and bars indicate standard deviations. Significant p-values determined by t-test are shown.

Figure 3. Graph shows levels of S100A7 in matched in-situ (DCIS) and invasive (INV) components within the 24/46 cases of DCIS associated with invasive carcinoma that showed expression in either component.

Table 1. Clinical-pathological features and frequency of S100A7 expression in DCIS

		DCIS-		DCIS+		All DCIS	
		#	%	#	%	#	%
ER	-ve	23	25%	19	42%	42	30%
	+ve	70	75%	26	58%	96	70%
Grade	1	36	38%	9	20%	45	32%
	2	33	35%	19	41%	52	38%
	3	24	27%	18	39%	42	30%
Necrosis	-ve	50	54%	15	33%	65	47%
	+ve	43	46%	31	67%	74	53%
s100a7	-ve	50	54%	22	48%	72	52%
	+ve	43	46%	24	52%	67	48%

Table 2. Relationship of S100A7 with prognostic factors in DCIS.

		S100A7		
		-ve	+ve	pvalue
ER	-ve	16	33	p=0.0008
	+ve	56	33	
Grade	1	37	8	
	2	26	26	p<0.0001
	3	9	32	
Necrosis	-ve	47	18	p<0.0001
	+ve	25	48	